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1 **The Varicella-Zoster virus IE4 protein: a conserved member of the herpesviral**
2 **mRNA export factors family and a potential alternative target in antiherpetic therapies**

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7 **Isabelle Ote, Jacques Piette and Catherine Sadzot-Delvaux***

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16
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21 **Abstract**

22 During a viral infection, in addition to cellular mRNAs, amounts of viral mRNAs have to be
23 efficiently transported to the cytoplasm for translation. It is now established that herpesviruses
24 encode a conserved gene family whose proteins act as viral mRNA export factors that mediate
25 nucleocytoplasmic transport of viral transcripts and eventually modulate through this
26 mechanism the antiviral response. This conserved family of proteins contains the IE4 protein
27 of Varicella-Zoster virus (VZV). Here, we compared the functional characteristics of IE4 with
28 those of its herpesviral homologues and proposed a model by which IE4 would be able to
29 recruit the essential TAP/NXF1 receptor to viral transcripts. Moreover, on the basis of their
30 crucial roles in the infectious cycle, these conserved viral factors should be considered as
31 alternative targets in therapeutic approaches. Here, we discussed the possibility of developing
32 antiherpetic agents targeting IE4 or its herpesviral homologues.

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36 **Key words:** VZV, IE4, herpesviral homologues, mRNA export, antiherpetic therapies

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41 **1. Introduction**

26 Herpesviruses are DNA viruses whose genome is transcribed in three successive phases
27 within the nucleus of the infected cells. Generally, in response to a viral infection, the host
28 immune system reacts and sets up an inflammatory response aiming to eradicate the viral
29 progression. Herpesviruses are known to induce such an innate immune response during acute
30 infection. In parallel, herpesviruses have also been described as being able to escape these
31 antiviral responses. In this perspective, herpesviruses encode proteins that are known to
32 interfere with the host immunity and whose expression was shown to be required to inhibit
33 some antiviral responses. Among these proteins, a conserved family of herpesviral proteins
34 has been described to interfere with the post-transcriptional gene regulation through the
35 cellular machinery of mRNA splicing, stability, export and translation. This family contains
36 the ICP27 protein of herpes simplex virus type 1 (HSV-1), the UL69 protein of human
37 cytomegalovirus (HCMV), and the EB2 protein of Epstein-Barr virus (EBV), respectively
38 alpha-, beta- and gamma-herpesviruses. The IE4 protein, encoded by the Varicella-Zoster
39 virus (VZV), another alpha-herpesvirus, also belongs to this conserved family and its
40 characteristics will be compared in this review with those of its homologues.

41

42 **2. The herpesviral mRNA export factors**

43 Structurally, HSV-1 ICP27, HCMV UL69, EBV EB2 and VZV IE4 share an overall amino
44 acid homology ranging between 20% and 30%. Moreover, they all contain, within their C-
45 terminal part, an homology region whose homology with ICP27 can be as high as 40% to
46 60% (Fig. 1) [1, 2]. Functionally, ICP27, UL69 and EB2 were all originally described as
47 *trans*-regulatory proteins and it was initially assumed that their stimulatory functions on gene
48 expression, from various viral as well as cellular promoters, occurred at the transcriptional
49 level. Progressively, this transactivation appeared to occur at the RNA level, suggesting a
50 post-transcriptional mechanism. Years later, it is now clearly demonstrated that these proteins

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share common characteristics, such as a nucleocytoplasmic shuttling activity, an RNA-binding domain (RBD) and the capacity to interact with cellular mRNA export factors, which identify them as viral mRNA export factors (Fig. 1). Available data suggested that IE4 also acts as an important regulator on VZV and cellular genes expression and could exert its functions at a post-transcriptional level. Although the molecular mechanisms are not yet fully characterized, we have recently shown that IE4 also shares the common characteristics of these herpesviral export factors [3].

3. Their nucleocytoplasmic shuttling activity

All members of this protein family have been reported to shuttle between the nucleus and the cytoplasm independently of virus-encoded cofactors indicating that it is an intrinsic characteristic [4-7]. Generally, nucleocytoplasmic protein trafficking occurs through direct interactions between the transport signals NLS and NES on the proteins and import or export receptors that mediate passage through the nuclear pore complex (NPC).

Nuclear localization signals (NLSs) have been described for the four homologues but they differ from each other (Fig. 2.). First, ICP27 was shown to possess a strong NLS, mapping to residues 110 to 137, which bears similarity with the bipartite NLS found in *Xenopus laevis* nucleoplasmin. Moreover, the sequence mapping to residues 110 to 152, including this strong NLS and the 15 contiguous residues which consist entirely of arginine and glycine residues, was demonstrated to function as nucleolar localization signal (NuLS) [8]. In contrast to ICP27, UL69 localizes within the nucleus but is excluded from the nucleolus [2]. It was also shown to contain an arginine-rich region at the N-terminus which resembles a bipartite NLS [6]. Similarly, EB2 was shown to contain two KH-rich motifs, mapping to residues 127-130 and 143-145, which function as two independent NLS sequences [9]. A basic arginine-rich

76 region, mapping to residues 130-143, was also described in the N-terminal part of IE4 and
77 was proposed to be good candidate for NLS [7].

78
79 Even more different nuclear export signals (NESs) are present in the sequence of these viral
80 proteins (Fig. 3). Most of the known shuttling proteins use a leucine-rich NES similar to that
81 of HIV-1 REV, which directly interacts with CRM-1 nuclear export receptor to be transported
82 into the cytoplasm. This CRM-1-dependent nuclear export can be blocked by the antibiotic
83 leptomycin B (LMB), which prevents the association of NES-containing proteins with CRM-
84 1 [10]. Whereas ICP27 and EB2 possess a leucine-rich NES located in their N-terminal part,
85 the requirement of CRM-1 for their shuttling has been much discussed. Since initial studies
86 suggested that a LMB-sensitive leucine-rich region could be involved in the
87 nucleocytoplasmic shuttling of EB2 [5, 11], later studies identified the unique sequence
88 responsible for the EB2 shuttling that was shown to be LMB-insensitive [9, 12]. Similarly,
89 although the export of ICP27 was first reported to be sensitive to LMB [13, 14], the leucine-
90 rich N-terminal sequence that is required for an efficient ICP27 export was further shown to
91 function as a CRM-1-independent NES [15, 16]. Singularly, a novel type of LMB-insensitive
92 export signal was identified within the UL69 unique C-terminal region [6]. This 28-amino
93 acid domain is not a leucine-rich one and the export pathway used by this unique NES has not
94 yet been defined. Whereas VZV IE4 exhibits a mainly cytoplasmic localization and should
95 bear a nuclear export signal, no classical NES sequence can be found within its N-terminal
96 region. However, a putative sequence rich in leucine residues can be seen into the central part
97 of the protein and could correspond to an NES, but the functionality of this putative signal has
98 never been demonstrated [7].

58 100 **4. Their RNA-binding domains**

101 Another characteristic of all known viral mRNA export factors is the presence of an RNA-
1 binding domain (Fig. 4.). ICP27 was shown to require an arginine-glycine-rich region,
2 102 binding domain (Fig. 4.). ICP27 was shown to require an arginine-glycine-rich region,
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4 103 mapping to residues 138 to 152, that constitutes an RGG-box RNA-binding domain [17].
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7 104 Recently, three arginine residues within this RGG box were shown to be methylated *in vivo*
8
9 105 but this methylation appears to regulate protein-protein interactions of ICP27 in the cytoplasm
10
11 106 rather than RNA binding [18]. In contrast, EB2 was demonstrated to harbour an RNA-binding
12
13 107 motif in a 33-amino acid segment that has the structural features of arginine-rich RNA-
14
15 108 binding motifs (ARMs) also found in many RNA-binding proteins [19]. This region contains
16
17 109 a short sequence rich in arginine and lysine residues, TRKQAR, which is characteristic of
18
19 110 ARMs. However, since this motif is not sufficient by itself to ensure RNA binding, additional
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21 111 amino acids present in the 33-amino acid segment are required for specific contacts with
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23 112 RNA. Likewise, three N-terminal arginine-rich motifs, termed R1, R2 and RS, are required
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25 113 for the RNA-binding activity of UL69 [20]. Both R1 and R2 show similarity with ARMs
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27 114 which have been identified in number of viral RNA-binding proteins and may function as a
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29 115 bipartite ARM. Removal of RS in combination with either R1 or R2 abolishes the RNA-
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31 116 binding activity of UL69 both *in vitro* and *in vivo*. Recently, we have shown that IE4 is able
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33 117 to bind directly to RNA *in vitro* and the three N-terminal arginine-rich domains Ra, Rb and
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35 118 Rc of IE4 were implicated in this interaction [3].
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46 120 Although one might assume that protein domains with numerous arginine residues bind non-
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48 121 specifically to RNA, sequence-specific interactions were demonstrated for the majority of the
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50 122 ARM-containing proteins investigated so far which includes the HIV-1 proteins REV and
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52 123 TAT or the phage λ N protein. Although there is no evidence for sequence-specific RNA-
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54 124 binding by ICP27, EB2 and UL69 *in vitro* [17, 19, 20], several data suggest the existence of a
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56 125 mechanism that confers specificity for the recognition of RNA *in vivo* (reviewed in [21]).
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126 Likewise, although the RNA-binding specificity of IE4 is still to be further explored,
127 preliminary data in infection suggest that the mechanism could be transcript- or gene class-
128 dependent [3]. Since all these herpesviral mRNA export factors interact with several viral and
129 cellular proteins, it is conceivable that some of these interactions may be needed to confer
130 specificity to RNA recognition. In addition, the secondary structure of RNA may be crucial
131 for its correct recognition.

132

133 **5. Their interactions with cellular mRNA export factors**

134 In metazoans, mature and well-processed mRNPs are transported into the cytoplasm by the
135 essential export factor TAP/NXF1. This mRNA export receptor shuttles between the nucleus
136 and the cytoplasm, and escorts competent mRNPs out of the nucleus through direct
137 interaction with nucleoporins lining the NPC [22]. Because of its low affinity for binding
138 mRNAs, TAP/NXF1 requires adaptor proteins to interface with mature transcripts ready for
139 export. So far, the best-characterized adaptor of TAP/NXF1 is the Aly/REF protein [23],
140 whose recruitment to mRNAs was shown to depend on the presence of UAP56 [24].
141 However, the observation that Aly/REF is dispensable for mRNA export in both *Drosophila*
142 and *C. elegans* suggests the existence of additional mRNA export adaptors [25, 26]. The
143 shuttling SR proteins have recently been proposed to act as adaptor of TAP/NXF1 and have
144 been shown to promote export of both intronless [27] and intron-containing [28] mRNAs.
145 Furthermore, SRp20 and 9G8 were shown to directly interact with TAP/NXF1 [28, 29] in a
146 phosphorylation-dependent manner [30, 31].

147

148 Thus, besides their ability to bind RNA, the capacity of the herpesviral proteins to interact
149 with factors of the cellular mRNA export machinery was also demonstrated. However, the

150 way by which these proteins interfere with the cellular mRNA export pathway is not unique
1
2 151 (Fig. 1).

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7 153 In one hand, ICP27 and EB2 were shown to interact with the mRNA export receptor
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9 154 TAP/NXF1 and its adaptor Aly/REF. The C-terminal leucine-rich region of EB2 was shown
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11 155 to be implicated in the interaction with Aly/REF *in vitro*. Aly/REF co-immunoprecipitates
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13 156 also with EB2 from transfected cells through interaction with the same region, but this
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15 157 interaction is RNase-sensitive suggesting that *in vivo* these proteins are part of a
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17 158 ribonucleoprotein complex stabilized by RNA [9]. This Aly/REF binding motif was shown to
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19 159 be required for both EB2-mediated mRNA export and production of infectious virions. In
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21 160 addition to its indirect recruitment via Aly/REF, TAP/NXF1 was also shown to directly
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23 161 interact with the N-terminal NES of EB2, containing several arginine-rich domains that
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25 162 cooperate to allow efficient interaction with TAP/NXF1 [32]. On its side, ICP27 was reported
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27 163 to interact with TAP/NXF1 either directly or through its interaction with Aly/REF. In infected
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29 164 cells, ICP27 was shown to relocate Aly/REF from sites of cellular splicing to sites of viral
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31 165 transcription [16]. This interaction with Aly/REF was first shown to occur through the ICP27
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33 166 region that contains the RNA-binding domain [15], and was later demonstrated to be RNase-
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35 167 sensitive, confirming that RNA also stabilizes the complex [9]. Direct interaction with
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37 168 TAP/NXF1 itself requires both ICP27 N-terminus and C-terminus [33], and was shown to be
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39 169 essential for both efficient export of viral transcripts and HSV-1 replication [34].
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53 171 On the other hand, UL69 has not yet been shown to interact with Aly/REF or TAP/NXF1.
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55 172 Instead, UL69 was demonstrated to directly bind to the highly related cellular DEXD/H-box
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57 173 RNA helicases UAP56 and URH49 [20, 35]. The interaction between UL69 and
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59 174 UAP56/URH49 was demonstrated both in transfected and infected cells, and it was excluded
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175 that RNA acts as bridging factor since the complexes are RNase-insensitive. Therefore, in
176 contrast to ICP27 and EB2, direct RNA-binding is dispensable for UL69 RNA export activity.
177 Comparatively, both UAP56 interaction and nucleocytoplasmic shuttling are prerequisites for
178 this UL69 function [20].

179
180 In the case of IE4, we showed that the protein interacts *in vivo* with the three shuttling SR
181 proteins SRP20, 9G8 and ASF/SF2 [3]. This interaction, which is not bridged by RNA, was
182 shown to occur through the N-terminal arginine-rich domains of IE4. Contrastingly, we
183 showed that the recruitment of TAP/NXF1 by IE4 is RNase-sensitive, which suggests that it
184 occurs through the SR proteins binding and that the presence of RNA stabilizes these
185 complexes.

186
187 Thus, although specificities exist, all the data suggest that the herpesviral mRNA export
188 factors use a general mechanism in which they bind to specific viral mRNAs and recruit these
189 transcripts to the cellular mRNA export TAP/NXF1 pathway through direct interaction with
190 the receptor TAP/NXF1 or with one of its adaptors such as Aly/REF, UAP56/URH49 or the
191 shuttling SR proteins.

192 193 **6. Their functions in the RNA export**

194 Very early, ICP27-mediated gene regulation was suggested to occur post-transcriptionally at
195 the level of RNA processing [36]. Few years later, ICP27 was suggested to be involved in the
196 nucleocytoplasmic RNA transport of virus intron-containing transcripts [37]. It was then
197 demonstrated that ICP27 binds specifically to viral intronless mRNAs in both cellular
198 compartments [38], indicating a direct role in the nuclear export of viral mRNAs. The
199 shuttling of ICP27 has been also reported to mediate the expression of some viral late genes

200 [39]. During the ten last years, the role of ICP27 in RNA export has been extensively
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2 201 documented (reviewed in [40, 41]). Recently, a microarray analysis has demonstrated that
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4 202 efficient export of the majority of viral transcripts requires that ICP27 be able to bind RNA
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7 203 and to interact with TAP/NXF1 [42]. Furthermore, the knock-down of TAP/NXF1 was shown
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10 204 to result in barely detectable level of two late proteins gB and gC, and in greatly reduced
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12 205 HSV-1 viral titers [34]. Very recently, the knock-down of SRp20 and 9G8 was also shown to
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14 206 reduce the virus growth, suggesting that these factors could play a role as HSV-1 mRNAs
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17 207 export adaptors and contribute to the overall export efficiency during HSV-1 infection [43].
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21 209 Similarly, EB2 has been described to lead to an increased cytoplasmic accumulation of many
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24 210 mRNAs, including those from reporter genes and those from a subset of EBV early genes
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26 211 coding for essential proteins involved in the EBV DNA replication during the productive
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29 212 cycle [44]. Furthermore, EB2 has been shown to be essential for the production of infectious
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31 213 viral particles [45] and for the nuclear export of most, but not all, late mRNAs produced from
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34 214 genes implicated in the capsid assembly and maturation [46].
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38 216 In contrast, while the required domains of UL69 and its cellular partners begin to be well
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41 217 defined, its RNA export activity has been only visualized in reporter assays [20, 35] and the
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43 218 viral targets of UL69 are still to be defined. Alternatively, since UL69 is also a constituent of
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46 219 HCMV virions, RNA-binding by UL69 might be involved in the packaging of RNAs into
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49 220 HCMV viral particles, which incorporate a subset of viral and cellular transcripts in the
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51 221 course of infection.
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55 223 Recently, we showed that IE4 also influences export of a reporter mRNA and that the
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58 224 knockdown of TAP/NXF1 prevents this IE4-mediated export [3]. Furthermore, we observed
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225 that TAP/NXF1 is also required for the correct expression of some VZV genes involved in
1 virions assembly. Thus, although the viral targets have to be further characterized, we
2 226 suggested that IE4, through its interaction with the SR proteins, is able to access the export
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4 227 TAP/NXF1 pathway to carry some specific VZV mRNAs to the cytoplasm.
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11 230 **7. Their functions in the RNA alternative splicing**

12 231 ICP27 was previously shown to post-transcriptionally stimulate mRNA accumulation from a
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14 232 transfected viral late gene encoding the glycoprotein C (gC) [47]. Further studying this
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16 233 observation, it was observed that the gC mRNA produced by ICP27 transactivation is an
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18 234 unspliced form, due to the retention of a previously undescribed intron from the gC transcript
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20 235 [48]. Contrastingly, during a viral infection, both transcripts and both proteins are detected,
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22 236 and it appears that HSV-1 has evolved to express two versions of the gC transcript. The
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24 237 spliced transcript encodes a variant of the glycoprotein C that lacks its transmembrane domain
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26 238 and that is efficiently secreted from infected cells, suggesting that it could function as a
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28 239 virulence factor. As ICP27 has been previously shown to inhibit cellular mRNA splicing
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30 240 during infection, this gC intron retention function could simply reflect a non-specific
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32 241 suppression of splicing. Alternatively, this ability of ICP27 may be a transcript- or intron-
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34 242 specific effect and not the result of a general inhibition of mRNA splicing. Although still to
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36 243 be confirmed, the gC gene could harbour a silencing element in its 5' half, distinct from the
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38 244 intron, and the effects of this sequence could be overcome by ICP27. The same authors also
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40 245 showed that IE4 lacks this ICP27 capacity of alternative splicing and is not able to promote
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42 246 HSV-1 gC intron retention [48].
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55 248 Also very recently, EB2 was shown to induce alternative splicing of a cellular RNA
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57 249 transcript, suggesting that viruses may also regulate cellular gene expression at the level of
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250 alternative splicing. As mentioned above, when expressed in EBV-negative cells, EB2 has a
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2 251 broadly inhibitory effect on cellular mRNA accumulation. Nevertheless, EB2 causes several
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4 252 cellular transcripts to accumulate at higher levels [49]. These transcripts include STAT1 and
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7 253 several interferon (IFN)-stimulated genes. The STAT1 protein is a central mediator of both
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10 254 type I (IFN- α/β) and type II (IFN- γ) IFN signal transduction pathways. It is expressed as two
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12 255 isoforms, STAT1 α and STAT1 β . STAT1 β mRNA is generated by cleavage and
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14 256 polyadenylation at an alternative site in the last intron of the STAT1 pre-mRNA, and, as the
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17 257 produced protein lacks the *trans*-activating domain encoded in the last exon, STAT1 β acts as
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20 258 a dominant-negative repressor of STAT1 α . EB2 was shown to disproportionately increase the
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22 259 relative amount of STAT1 β mRNA [49]. More precisely, EB2 was identified to induce the
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25 260 alternative splicing of STAT1 at a novel 5' splice site (Alt 5'SS), resulting in a STAT1 α'
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27 261 mRNA incapable of producing STAT1 α (Fig. 5A.) [50]. EB2 was shown to act as a splicing
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30 262 factor by directly binding to the same STAT1 RNA region as ASF/SF2 and to compete with
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33 263 this splicing factor in the splice site selection. By increasing the STAT1 β /STAT1 α RNA
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35 264 ratio, EB2 changes the relative levels of ISGs depending on how responsive they are to
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38 265 STAT1 β , which affects cellular apoptosis and resistance to viral infection. As we showed that
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40 266 IE4 directly interacts with ASF/SF2, we here wondered if IE4 also shares this novel EB2
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43 267 function. We used the STAT1 minigene construct for *in vivo* splicing assay (Fig. 5B). HeLa
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45 268 cells were co-transfected with this reporter plasmid and an IE4- or its homologues- encoding
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48 269 vector. Total RNAs were extracted and STAT1 transcripts were observed by RT-PCR using
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50 270 specific primers amplifying both alternatively spliced products (α or α'). We showed that
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52 271 EB2 is the unique homologue able to alternatively splice this STAT1 gene, and that the
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55 272 presence of either IE4, or ICP27 and UL69, only leads to the constitutively spliced STAT1
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57 273 mRNA.
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275 8. Their regulation by phosphorylation

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2 276 As these four proteins are known to be phosphoproteins, it had been speculated that their
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4 277 activities could be regulated through phosphorylation. Very recently, two studies
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7 278 simultaneously highlighted a link between the cellular CDKs activity and the UL69
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10 279 localization and functionality [51, 52]. Furthermore, the HCMV protein kinase UL97, which
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12 280 had been characterized as a CDK-related kinase, was also shown to modulate the nuclear
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14 281 localization and activity of UL69. UL69 was shown to physically interact with UL97 and to
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17 282 co-localize with CDK9. In addition, UL69 acts as a specific substrate in *in vitro* kinase
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19 283 reactions with the viral kinase UL97 and all the CDKs analyzed (CDK-1, -2, -7 and -9).
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22 284 Interaction studies showed that UL97 interacts with the UL69 N-terminus, which consists of
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24 285 multiple overlapping functionally important regions, such as the NLS, the UAP56/URH49
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27 286 interaction motif and the RNA binding site, as well as with the UL69 C-terminus, which
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29 287 contains the NES. Therefore, phosphorylation within these domains may trigger a fine-
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32 288 regulation of the distinct properties of UL69. Indeed, UL97-mediated phosphorylation of
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34 289 UL69 was demonstrated to enhance the latter's mRNA export activity, and protein kinase
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36 290 inhibitors, such as Gö6976 (UL97) and roscovitine or R58 (CDKs), significantly reduce this
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39 291 activity. Thus, cellular CDKs and viral UL97 activities are required to modulate the
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41 292 intranuclear localization and mRNA export function of UL69 during HCMV replication.

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46 294 Similarly, it has been shown that the protein kinase CK2 α and β subunits interact *in vitro* both
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49 295 individually, and more efficiently as a complex, with EB2 N-terminal and C-terminal domains
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51 296 [53]. Furthermore, EB2 was shown to co-immunoprecipitate from living cells with CK2. This
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54 297 latter was also shown to phosphorylate several residues within EB2 N-terminus, including the
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56 298 Ser-55, -56, and -57, which localize next to the EB2 NES. The phosphorylation mutant
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58 299 EB2S3A was shown to be unable to trans-complement EB2 in cells harboring an EB2-

300 defective EBV genome. This defect is not due to an impaired nucleocytoplasmic shuttling but
301 associated with a decreased cytoplasmic accumulation of several late viral mRNAs. These
302 results suggest that the CK2-mediated EB2 phosphorylation is crucial for the production of
303 EBV infectious virions.

304
305 ICP27 has also been demonstrated to be phosphorylated on serine residues during infection.
306 Two serine residues at positions 16 and 18 within the leucine-rich N-terminal region required
307 for export were shown to be phosphorylated by CK2, and a serine at position 114 within the
308 NLS was identified to be targeted by PKA [54]. As the S114A mutation diminishes the ICP27
309 import into the nucleus, it was suggested that the phosphorylation by PKA may regulate the
310 ability of ICP27 to interact with importins. All ICP27-phosphorylation mutant viruses were
311 also shown to reach lower growth curves compared to wild type HSV-1, further suggesting a
312 role of phosphorylation in the various ICP27 functions during viral infection, including its
313 shuttling and RNA export activities (reviewed in [41]). Recently, the ICP27 phosphorylation
314 mutants at Ser-16, -18 and -114 residues were shown to display altered functional interactions
315 with Aly/REF and TAP/NXF1, although they are still able to bind HSV-1 mRNAs [55].
316 Interestingly, these mutants were also described to be defective in HSV-1 replication and viral
317 gene expression [56].

318
319 SRPK1 is the main serine/threonine-protein kinase that specifically phosphorylates the SR
320 proteins [57]. Through these phosphorylation events, SRPK1 regulates the localization and
321 functions of the SR proteins, as well in constitutive and alternative splicing as in mRNAs
322 export and translation (reviewed in [58]). As already mentioned, ICP27 was shown to interact
323 with SRPK1, as well as with SRp20 and other SR proteins [59]. ICP27 is also phosphorylated
324 by this kinase and several explanations for the ICP27-mediated SR protein hypo-

325 phosphorylation have been suggested. ICP27 could simply compete with SR proteins for the
1
2 326 SRPK1 binding, could mask potential phosphorylation sites in SR proteins, or could alter the
3
4 327 SRPK1 conformation resulting in changes of its activity. These possibilities are not mutually
5
6
7 328 exclusive and all result in splicing inhibition by impairing spliceosome assembly.
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12 330 Similarly, we showed that IE4 interacts with SRPK1, mainly through its Rb domain [3]. We
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14 331 also showed that the Ser-136 residue located in this region is phosphorylated by the kinase.
15
16 332 However, we were not able to demonstrate the role of this phosphorylation in the export
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18 333 activity of IE4. It could be implicated in the nuclear reimport of IE4, like it has been
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20 334 suggested for the SR proteins [30], or even be involved in the regulation of other still
21
22 335 unknown functions of IE4. The *in silico* analysis of IE4 also predicts many phosphorylation
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24 336 sites for the CK2, PKA and PKC kinases, but no role of these potential phosphorylation
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26 337 events on the IE4 activities has been studied so far.
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34 339 **9. The IE4-mediated recruitment of the TAP/NXF1 receptor**

35
36 340 SRp20 and 9G8 were shown to recruit TAP/NXF1 through a small arginine-rich peptide next
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38 341 to their RNA recognition motifs (RRMs) rather than through their C-terminal RS domains
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40 342 [29]. Although it was not determined, it is convincing to think that, in absence of RNA, the
41
42 343 interaction between IE4 and the SR proteins occurs through the Ra, Rb, Rc domains of IE4
43
44 344 and this small peptide (Fig. 6A.). By contrast, in presence of RNA, the RNA-binding domains
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46 345 of these proteins would preferentially interact with the transcript, leading to a conformation
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48 346 change of the SR proteins and allowing the small peptide to recruit TAP/NXF1 (Fig. 6B.).
49
50 347 Interestingly, we showed that the C-terminal cystein-rich region of IE4 is also required for its
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52 348 export activity since the IE4 Δ cys mutant was shown to be incompetent in a reporter export
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54 349 assay [3]. Since IE4 seems to dimerize through this C-terminal region, the dimerization of IE4
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350 could be a prerequisite to its function. However, it was shown that ICP27 interacts with
1
2 351 TAP/NXF1 via its C-terminus, which contains a zinc finger-like domain (CCHC) [33]. Such a
3
4 352 potential zinc finger domain is also present in the C-terminal region of IE4 [7]. We could thus
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6
7 353 hypothesize that the presence of RNA and the recruitment of TAP/NXF1 by the SR proteins
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10 354 would allow the C-terminal part of IE4 to bind TAP/NXF1, and that only these tripartite
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12 355 complexes would be competent for export (Fig. 6C.). This model would support our data
13
14 356 showing that the recruitment of TAP/NXF1 by IE4 is RNase-sensitive. Furthermore, IE4 was
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17 357 shown to be essential for VZV replication and more precisely the GKYFKC motif contained
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19 358 in the IE4 C-terminal region was shown to be crucial [60]. Thus, the role of IE4 as export
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22 359 adaptor and its capacity to access the TAP/NXF1 pathway could be indispensable for the
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24 360 VZV infection.

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362 **10. IE4 and its homologues as alternative targets in antiherpetic therapies**

363 So far, majority of the antiherpetic agents are based on modified nucleoside analogues such as
364 364 the currently available drugs acyclovir and gancyclovir. These drugs are competitively
365 365 incorporated into viral DNA, what further blocks the DNA polymerase activity and inhibits
366 366 the viral replication. However, over the past few years, the long-term usage of these antiviral
367 367 agents led to the emergence of nucleoside-analogue-resistant herpesvirus strains, resulting in
368 368 ineffective conventional therapies (reviewed in [61]). Additional antiherpetic treatments, such
369 369 as various antisense-mediated strategies, are therefore under development, in order to
370 370 specifically knockdown expression of some viral proteins.

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372 On the basis of their crucial roles in the infectious cycle, the viral export factors described
373 373 here must be considered as targets in these novel therapeutic approaches. Recently, the
374 374 antiviral activity of peptide-conjugated phosphorodiamidate morpholino oligomers (PPMO)

375 was investigated in HSV-1 ocular infection [62]. PPMO targeting ICP27 were shown to
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2 376 inhibit viral replication both in cell cultures and in eyes of mice. Since the antisense technique
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4 377 is in theory unlimited and that these PMO present pharmacological advantages compared to
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7 378 other antisense oligonucleotides, this novel technology could be further developed as
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9 379 alternative antiherpetic therapy.
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14 381 In addition to target specific viral proteins, several recent studies suggest to exploit the
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17 382 cellular proteins as potential targets for the development of novel antiviral agents. Targeting a
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19 383 host factor may be a useful tool to avoid the emergence of mutant resistant strains. However,
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21 384 inhibition of multifunctional cellular proteins could more largely deregulate the host cell
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24 385 signalling pathways and might result in significant undesirable effects. In the mRNA export
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26 386 pathway, rather than targeting the essential TAP/NXF1 receptor, the safest strategy would be
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29 387 to knockdown UAP56 or the shuttling SR proteins, which are the specific cellular proteins
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31 388 targeted by UL69 or IE4 in HCMV and VZV infection respectively [3, 35]. Similarly, the
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34 389 specific knockdown of the DEAD-box RNA helicase DDX3 was shown to inhibit HIV-1
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36 390 replication without affecting cell viability [63].
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41 392 In addition to inhibit expression of these viral export factors, or expression of their cellular
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43 393 partners, antiviral drugs targeting their specific functions and regulation could also be
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46 394 developed. As all these proteins are regulated by phosphorylation, kinase inhibitors are
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48 395 promising key agents in antiviral therapies. Thus, similarly to roscovitine that was shown to
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51 396 reduce CDKs-mediated UL69 activity [51], specific inhibitors of CK2 or SRPK1 could
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53 397 potentially reduce ICP27, IE4 or EB2 export functions. Furthermore, viral kinases also
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55 398 represent potential targets in antiherpetic treatments. For example, maribavir, a selective
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399 inhibitor of the UL97 kinase [64], is currently under phase III clinical trial to treat HCMV
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7 402 Finally, example could be taken from the prototypical viral export factor HIV-1 REV protein,
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9 403 which has already been targeted in clinical trial. Indeed, in the anti-HIV gene-therapy study,
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11 404 the lentiviral vector transduction of a dominant-negative REV protein was shown to potently
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13 405 inhibit HIV-1 replication [65]. Thus, various mutants of the herpesviral export factors,
14
15 406 impaired in their capacity of either nucleocytoplasmic shuttling, RNA-binding, or cellular
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17 407 proteins interaction, could be tested in their ability to act as dominant-negative inhibitors, thus
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19 408 corresponding to good candidates of protein-based agents in alternative antitherpetic therapies.
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Fig. 1. Organization of the different functional domains of the herpesviral homologues. ICP27 of the herpes simplex virus type-1 (HSV-1) from the alpha-herpesvirus subfamily. UL69 of the human cytomegalovirus (HCMV) from the beta-herpesvirus subfamily. EB2 of the Epstein-Barr virus (EBV) from the gamma-herpesvirus subfamily. IE4 of the Varicella-Zoster virus (VZV) from the alpha-herpesvirus subfamily. NES: nuclear export signal; NLS: nuclear localization signal; RBD: RNA-

596 binding domain; TAP/NXF1, Aly/REF, UAP56, SRPK1 and SR proteins: binding sites of the
 1 597 respective cellular proteins; IE4 and UL97: binding sites of the respective viral proteins. The
 2 598 phosphorylation sites discussed in the text are shown by black stars.
 3 599

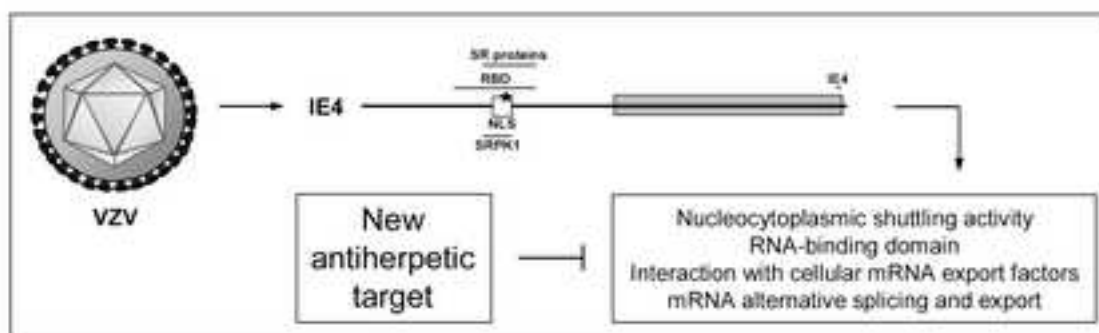
4 600 **Fig. 2. Comparison of the different nuclear localization signals (NLSs) sequences.** The HSV-1
 5 601 ICP27 NLS bears similarity with the bipartite *Xenopus laevis* nucleoplasm NLS (underlined). The
 6 602 HCMV UL69 NLS is an arginine-rich (indicated in bold) region which resembles a bipartite NLS. The
 7 603 EBV EB2 NLS contains two independent KH-rich motifs (underlined). The VZV IE4 NLS was
 8 604 proposed to correspond to the basic arginine-rich (indicated in bold) region mapping to residues 130-
 9 605 143. Numbers refer to the positions of the amino acid sequences with each protein.
 10 606

11 607 **Fig. 3. Comparison of the different nuclear export signals (NESs) sequences.** The HSV-1 ICP27
 12 608 NES is a leucine-rich NES similar to the HIV-1 REV NES. The conserved amino acids are shown in
 13 609 bold. The HCMV UL69 NES is 28-amino acid domain whose essential residues are indicated is bold.
 14 610 The EBV EB2 NES is a leucine-rich region mapping to residues 61-140. A leucine-rich motif found
 15 611 into the central part of VZV IE4 could correspond to an NES. Numbers refer to the positions of the
 16 612 amino acid sequences with each protein.
 17 613

18 614 **Fig. 4. Comparison of the different RNA-binding domains (RBDs) sequences.** The HSV-1 ICP27
 19 615 RNA-binding domain is constituted by a RGG-box. All arginine residues are indicated in bold and the
 20 616 three methylated arginine residues are marked by asterisks. The HCMV UL69 RNA-binding domain
 21 617 contains three arginine-rich (indicated in bold) motifs, termed R1, R2 and RS (underlined). The EBV
 22 618 EB2 RNA-binding domain is arginine-rich (indicated in bold) region that contains a short sequence
 23 619 (underlined) characteristic of ARMs. The VZV IE4 RNA-binding domain maps to the three arginine-
 24 620 rich (indicated in bold) motifs, termed Ra, Rb and Rc (underlined). Numbers refer to the positions of
 25 621 the amino acid sequences with each protein.
 26 622

27 623 **Fig. 5. IE4 and its homologues in the alternative splicing of the STAT1 RNA.** (A) Schematic
 28 624 representation of the STAT1 minigene (adapted from [50]) which contains STAT1 exon 23, exon 24
 29 625 and intervening sequence. Alternative processing events generate either the α or the α' spliced form,
 30 626 in which the α 5' donor site is skipped and the α' 5' donor site is used (Alt5'SS). (B) HeLa cells were
 31 627 transfected with the STAT1 minigene and expression plasmids as indicated (lanes 2 to 6).
 32 628 Untransfected cells were used as a negative control (lane 1). Total RNAs were isolated from
 33 629 transfected HeLa cells by TriPure/chloroform (Roche) extraction and isopropanol precipitation. A
 34 630 DNase treatment with DNase I recombinant RNase free (Roche) was performed according to the
 35 631 manufacturer's protocol to digest any DNA contamination from RNA preparations. Reverse
 36 632 transcription was performed with 1 μ g of purified total RNAs by use of M-MLV Reverse
 37 633 Transcriptase and Random Primers (Invitrogen) for 1 h at 37°C. PCRs were performed on 2 μ l of
 38 634 cDNAs using GoTaq DNA polymerase (Promega) and specific primers (STAT1_FW 5'-
 39 635 TGGAAAGCGTACACAATGTGT and STAT1_RV 5'-CATACTGTCTCGAATTCTACAGA)
 40 636 amplifying both alternatively spliced products (α or α'). The PCR-amplified products were then
 41 637 analysed on 1% agarose gel and visualized by ethidium bromide staining. Reverse transcription
 42 638 without reverse transcriptase (-RT, lane 7) was performed as purity control of our RNAs and GAPDH
 43 639 PCR was used as input control (GAPDH_FW 5'-CATCACTGCCACCCAGAAGACTGTGGA and
 44 640 GAPDH_RV 5'-TACTCCTTGGAGGCCATGTAGGCCATG). The expression of the different
 45 641 proteins was verified by Western Blotting using anti-V5 and anti-Flag antibodies.
 46 642

47 643 **Fig. 6. Model of recruitment of TAP/NXF1 by IE4 in a SR proteins- and RNA-dependent way.**
 48 644 (A) In absence of RNA, IE4 and the SR proteins interact through the Ra, Rb, Rc domains of IE4 and a
 49 645 small peptide next to the RRM of SR proteins. (B) In presence of RNA, a conformation change of the
 50 646 SR proteins allows the recruitment of TAP/NXF1. (C) IE4 binds TAP/NXF1 through its C-terminus
 51 647 leading to a tripartite export-competent complex.
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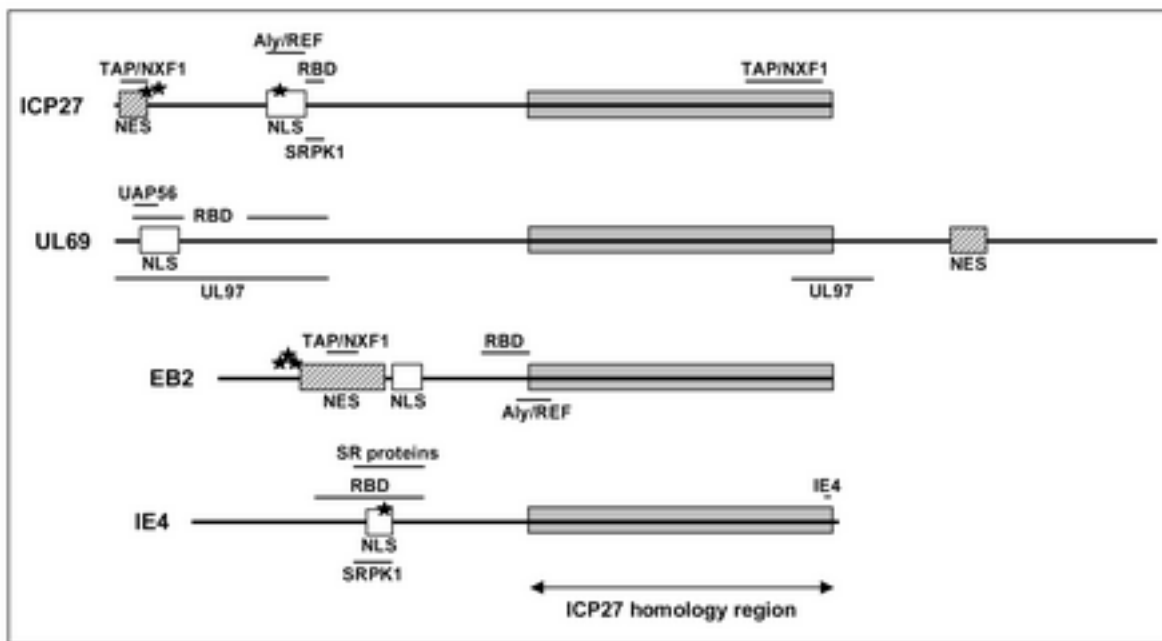


Fig. 1.

Nucleoplasmin	154- <u>K</u> RPAA <u>T</u> KKAGQA <u>K</u> KKK -171
HSV-1 ICP27	109- <u>A</u> RRPSCSPERHG <u>G</u> KVAR <u>L</u> QPPPTKAQPA -138
HCMV UL69	18- ERERRARRARRFCLDYEPVPRKFRRERS -47
EBV EB2	126- <u>K</u> RRRGEVHGCTDESYG <u>K</u> RR -146
VZV IE4	129- RKHRDRSLSNRRRR -144

Fig. 2.

HSV-1 ICP27	4- IDMLIDLGLDL -16
HCMV UL69	596- APPAQPPSQPQQHYSEGELEEEDESDDA -625
EBV EB2	60- DEDPTPAHAIPARPSSVITPTSASFVIPRKKW DLQDKTVTLHRSPLCRDEDEKEETGNSSYTR GHKRRRGEVHGCTDES -141
VZV IE4	320- LLENLKLKLG -331

Fig. 3.

HSV-1 ICP27	137- <u>RGGRRGRRRGRGRGG</u> -153 * * *
HCMV UL69	16- <u>LSEERRARRARRE</u> FLDYEPVPRKFRERSPTSPSTRN GAAASEYHLAEDTVGAASHHHRPCVPARRPRYSKDDDT EGDPDHYPPLPPSSRHALGGTGGHIIMGT <u>AGFRGGHR</u> <u>ASSFKRRV</u> -140
EBV EB2	189- <u>RSTRKQARQERSQRPLPNKPWF</u> DMSLVKPVSKIT -224
VZV IE4	70- <u>RYSKRRHGRRESYHHNRPKTLVVLPDSNHHGGRD</u> VET <u>GYARIERGHRRSSRSYNTQSSRKHRDRSLSNRRRRPTTP</u> PAMTTGERNDQTHDESY <u>RLRFSKRDARRERIRK</u> -181

Fig. 4.

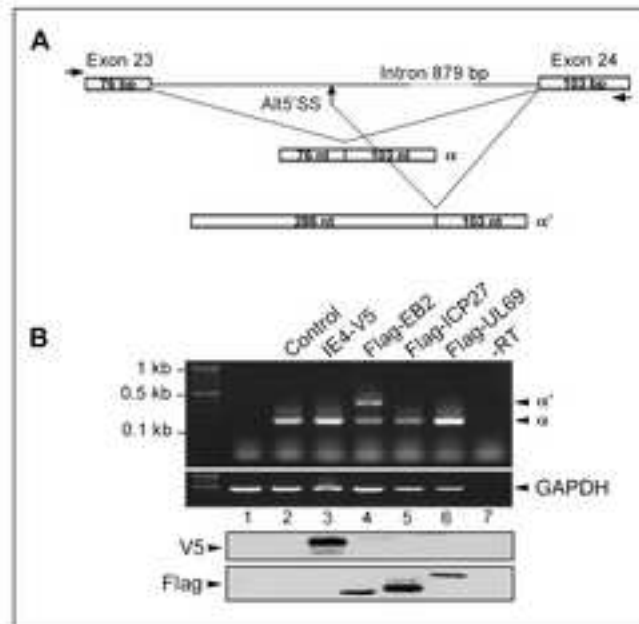


Fig.5.

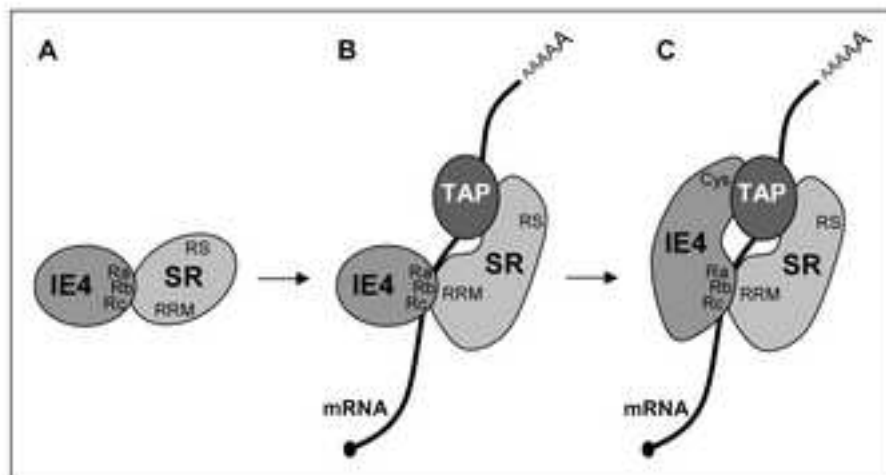


Fig. 6.