Analysis of the Structure−Activity Relationship of Four Herpesviral UL97 Subfamily Protein Kinases Reveals Partial but not Full Functional Conservation†

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Herpesviral protein kinases of the UL97 subfamily are expressed by all known herpesviruses but the degree of functional conservation is unclear. A selection of representative members was investigated by a comparative structural and functional analysis. The coding sequences of human cytomegalovirus (HCMV) pUL97, rat CMV pR97, Epstein−Barr virus BGLF4, and herpes simplex virus UL13 showed a low degree of amino acid identity. A computational approach employing fold recognition techniques revealed structural similarity to the cellular kinase Cdk2 with a high level of conservation of the functionally important residues in ATP binding sites and the catalytic centers. Analyses of *in vitro* activities of these herpesviral protein kinases, including measurements of phosphorylation of cellular substrates, trans-complementation experiments with a UL97-deleted HCMV mutant, and sensitivity profiles toward protein kinase inhibitors, demonstrated marked similarities between pUL97 and pR97 and to a lesser extent between pUL97 and BGLF4 or UL13. Thus, the structure−activity analysis of pUL97-like herpesviral protein kinases indicates a partial but not a full conservation of their functional properties among the herpesviruses.

Introduction

Herpesviral gene expression is regulated by numerous interactions between viral and cellular proteins. The modulation of protein activity by posttranslational modification such as phosphorylation is one of the main regulatory effector pathways. Hereby, protein kinases either of cellular or viral origin play an important modulatory role within herpesviral replication cycles. Herpesviral genomes encode their own serine/threonine-type protein kinases which fulfill multiple functions and often are able to phosphorylate viral as well as cellular substrate proteins. Two subfamilies were defined for herpesviral protein kinases: the US3 subfamily (also termed HvUS, according to the kinase US3 of herpes simplex virus type 1 [HSV-1]) and the UL97 subfamily (also termed HvUL, according to the UL97-encoded kinase [pUL97][1] of human cytomegalovirus [HCMV[a]]). For HCMV pUL97, regulatory functions were assigned to at least three stages of viral replication, i.e., viral genome synthesis,[1−3] modulation of cellular factors involved in transcription and translation,[4−5] and nuclear egress of viral capsids.[6−7] Some of the cellular proteins phosphorylated by pUL97 were also identified as substrates of the homologous protein kinases of other herpesviruses. For example, eukaryotic translation factor EF-1δ and histone 2B seem to be common substrates of pUL97-related herpesviral protein kinases.[8−10] This indicates a functional similarity between the members of the UL97 subfamily, which, however, is not fully understood so far. These protein kinases appear to possess variable importance for viral replication: while UL13 is dispensable for growth of HSV-1 in cultured cells,[11] pUL97 is required for a high efficiency of HCMV replication.[12] A comparative analysis of four herpesviral protein kinases, performed on the levels of sequence analysis, prediction of protein structures, kinase activities, and complementation properties reveals novel aspects which may contribute to a better understanding of their natural functions. General interest may particularly be attracted by the kinases' inhibitor sensitivity profiles which were characterized both by experimental and computational approaches, illustrating the practical usefulness of sequence modeling in antiviral research.

Results and Discussion

Structural Features of Herpesviral Protein Kinases. Four open reading frames (ORFs) of the UL97 subfamily of herpesviral protein kinases were analyzed in this study for sequential conservation and showed low degree of 11.0% to 30.1% identity at the amino acid level (Table 1). The highest level of identity was observed between the human and rat cytomegaloviral protein kinases HCMV pUL97 and RCMV pR97 (30.1% for the total sequences and 44.7% for the kinase domains, respectively.[8,13] Sequence similarity to other protein families was beyond the limit of detection for conventional search tools. More sophisticated sequence analysis tools, such as profile-based methods or fold recognition (threading) techniques, that were accessible via a Structure Prediction Meta Server,[14] however, clearly demonstrated a remote homology to cellular serine/threonine-type protein kinases. The overall sequence identities between herpesviral and cellular protein kinases were low (5.1−12.5%; Table 1), but functionally important sequence motifs and residues within the kinase subdomains (SD) I to XI were considerably well conserved (Figure 1A). As described for many

† Protein data bank: UL97, AAV49323; R97, AAF99187; UL13, BAA84007; BGLF4, CAA24828; Cdk2, CAA43985; c-Src, NP_004374; p38, AAB66313; PKC, CAA36718.

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[a] Abbreviations: HCMV, human cytomegalovirus; RCMV, rat cytomegalovirus; EBV, Epstein-Barr virus; HSV-1, herpes simplex virus type 1; AD169delUL97, UL97-deleted HCMV; Ad, adenovirus; HFF, human foreskin fibroblasts; REF, rat embryonic fibroblasts; F, FLAG tag; HA, hemagglutinin tag; GST, gluthatione S transferase; RFP, red fluorescent protein; H2B, histone 2B.

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Table 1. Sequence Identity between Herpesviral and Cellular Protein Kinases

	Total Amino Acid Sequences [% identity][a]							
	UL97	R97	UL13	BGLF4	Cdk2	c-Src	p38	PKC α
UL97	100	30.1	12.2	11.0	5.4	10.4	6.6	11.4
R97		100	17.9	14.4	5.1	11.3	8.8	12.0
UL13			100	13.7	8.1	12.5	9.6	11.2
BGLF4				100	9.3	10.4	11.9	10.5
Cdk2					100	13.1	29.2	10.1
c-Src						100	11.1	11.9
p38							100	8.8
PKC α								100

	Sequence of ORF UL97 Finase Domain [% identity][b]							
	UL97 (329-572)	R97 (234-479)	UL13 (149-396)	BGLF4 (76-326)	Cdk2 (2-205)	c-Src (41-287)	p38 (19-289)	PKC α (110-360)
UL97 (329-572)	100	44.7	15.3	19.9	11.7	10.1	12.5	10.4

[a] Degrees of identity of the full-length sequences were derived from pairwise alignments between the herpesviral genes and cellular protein kinases Cdk2,[42] p38,[43] PKC α,[44] and c-Src (NCBI accession NP_004374).[45] [b] Essential parts of the protein kinase domains as defined by SDs I to IX of ORF UL97 and the respective homologous regions of other kinases were compaired by pairwise alignments.

members of the eukaryotic protein kinase superfamily, these regions are required for ATP binding and for the formation of the catalytic center.[15,16]

A close structural similarity was suggestive for SD I between all analyzed herpesviral protein kinases. SD I contains the glycine-rich motif GXXGXG (phosphate-binding P loop) which is nearly invariant and essential for ATP binding.[13] The invariant lysine of SD II and the nearly invariant glutamate of SD III, which are important for catalytic activity,[15] were also well conserved in the herpesviral protein kinases. The only variation was a conservative replacement of glutamate by aspartate in SD III of R97, which was expected to have only little effect on protein function. SDs IV and V are in general mainly formed by hydrophobic residues in cellular Ser/Thr kinases. Consequently, the polarity of the side-chains was found to be conserved in these SDs, but not the exact amino acid sequence. This fact may explain why the sequence similarity between the herpesviral and cellular Ser/Thr kinases was below the limit of detection for the respective SDs. In contrast, SDs VI to IX of herpesviral protein kinases were again quite well conserved. The only notable exception was the nonconservative replacement of glutamate by alanine in SD VIII of UL13. Since the region is suggested to play an important role in the recognition of peptide substrates, this exchange might possibly result in altered substrate binding properties of UL13. SD X was previously found to be quite poorly conserved even among cellular Ser/Thr kinases,[15] and thus the lack of sequence similarity in this region between cellular and viral protein kinases analyzed in our study was not unexpected. The nearly invariant arginine of SD XI, again was quite well conserved in all herpesviral protein kinases.

Based on the similarity of fold and functionally conserved residues between viral and cellular kinases, a molecular modeling of the viral kinases was initiated using the cellular protein kinase Cdk2 as a template (Figure 1B). Cdk2 (PDB 1vyw_C,[17]) was identified as the most suitable template among all available kinase gene sequences by performing a sequence and secondary structure analysis in Structure Prediction Meta Server (see Methods). The accuracy of the resulting models is generally limited by the low sequence identity between target and template. Modeling was therefore rather intended to confirm the overall fold than to address structural details (conclusions need to be drawn carefully; see description of Figure 1E−H below). A modeling of the kinase domain including SD I to IX was possible for pUL97 and pR97 (Figure 1C,D). The models indicated that the overall topology of the cellular Ser/Thr kinases and the location of the functionally important SDs is rather conserved in pUL97 and pR97 and that structural differences are mainly observed for the length and orientation of the loops connecting these domains. In contrast, no reliable modeling was possible for the C-terminal helices in BGLF4 (Epstein−Barr virus, EBV) and UL13 (herpes simplex virus type 1, HSV-1) due to the lack of a sufficiently high sequence similarity to Cdk2. For UL13, structural modeling stopped with SD VIII indicating differences in its C-terminal portion of the kinase domain. Interestingly, in the case of pUL97, this C-terminal portion of the kinase domain is essentially required for ganciclovir (GCV) phosphorylation and other activities.[7,18] Combined these findings pointed to a similarity of structural features between herpesviral protein kinases: a pronounced structural similarity was likely for the two cytomegaloviral kinases pUL97 and pR97, while more differences toward pUL97 were predicted for UL13 and BGLF4.

Functional Properties of Herpesviral Protein Kinases: Protein Interactions and Substrate Phosphorylation. pUL97, pR97, UL13, and BGLF4 were transiently expressed in 293 cells in a catalytically active form. *In vitro* kinase assays revealed typical activities for all four protein kinases, such as autophosphorylation and histone 2B (H2B) phosphorylation (Figure 2A). Histones are common *in vitro* substrates of a variety of eukaryotic protein kinases and H2B is a known cellular substrate of many herpesviral protein kinases.[10,19] In this approach, all analyzed protein kinases were positive and BGLF4 showed a particularly high activity in H2B phosphorylation (Figure 2A, lane 5). Variations in the levels of recombinant expression were demonstrated (Figure 2A, lanes 2−5) and confirmed by parallel setups (data not shown), thus leading to a limitation in the possibility of a quantitative evaluation.

In addition, the cellular protein p32 was recently identified as an interactor of pUL97 which is phosphorylated by pUL97 as a recombinantly or endogenously produced substrate.[7] Here we demonstrated that, in addition to pUL97, also pR97 and BGLF4 were able to phosphorylate recombinant GST-p32, albeit at a lower level than pUL97, while UL13 lacked this activity (Figure 2B, lanes 4−7). A catalytically inactive pUL97 mutant, K355M, was used to exclude the possibility that another kinase, incidentally coimmunoprecipited, was responsible for phosphorylating p32. The lack of phosphorylation of p32 by immunoprecipitates containing mutant K355M (in contrast to the positive result with wild-type pUL97) demonstrated that p32 is a direct substrate of pUL97 (Figure 2B, lane 8). Using coimmunoprecipitation assays, the direct interaction with endogenous p32 was demonstrated for FLAG (F)-tagged versions of pUL97, pR97 and, at a lower level, BGLF4; UL13 showed no interaction

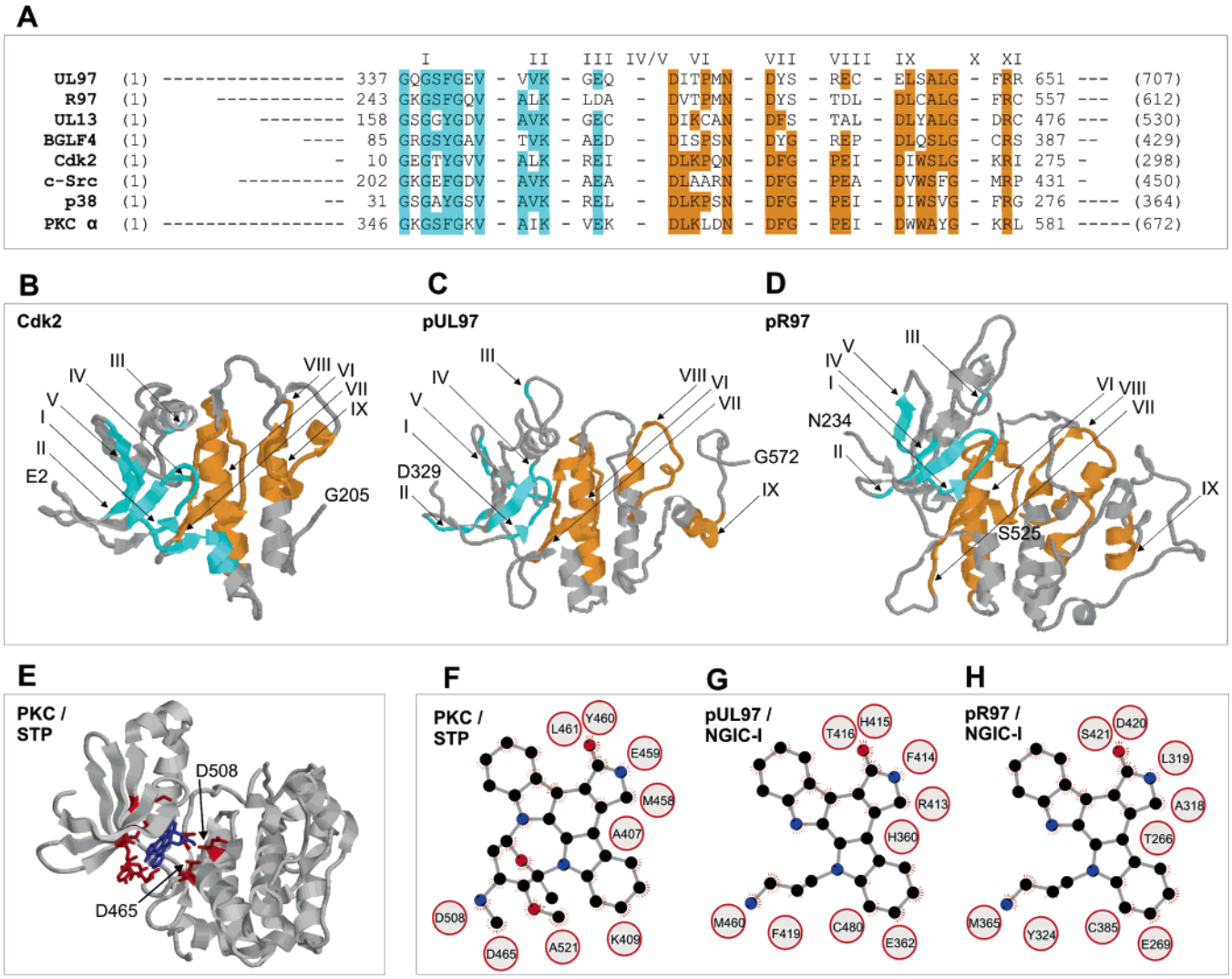

Figure 1. Sequence and structure informations based on a comparison between herpesviral and cellular protein kinases. (A) Sequence alignment of herpesviral and cellular protein kinases. The conserved protein kinase subdomains (SD) I to XI[15,16] of the herpesviral protein kinases were identified by multiple and pairwise alignments to a series of cellular protein kinases. A schematic alignment was generated for four cellular and four herpesviral protein kinases, including SDs I to XI and intermittent sequence stretches as marked by hyphens. (B−D) Predicted three-dimensional structures of two herpesviral protein kinase domains. HCMV pUL97 and RCMV pR97 were modeled on the template Cdk2 (PDB 1vyw chain C). SDs I to V are marked in cyan (putative ATP binding site) and SDs VI to IX in orange (putative catalytic site). (E) Schematic presentation of the PKC θ structure (gray) with the inhibitor STP (blue). Residues which contact the ligand and are not conserved in the viral protein kinases are shown as red sticks. Sequence positions 465 and 508 which contact the glycosidic moiety of STP are labeled. (F) Schematic presentation of the contacts between the "nonconserved" residues of PKC and STP. Residues of PKC are shown as red circles, and STP is shown in stick presentation. The ligand is shown in ball-and-stick-presentation with carbon, oxygen, and nitrogen atoms colored in black, red, and blue, respectively. (G, H) Schematic presentation of the contacts between NGIC-I and pUL97 or NGIC-I and pR97, respectively. Residues of the kinases are shown as red circles, and NGIC-I is shown in stick presentation. Amino acids found at equivalent sequence positions are printed on identical positions in panels F to H. For chemical structures of the inhibitors, see reference 9.

with p32 (Figure 2C, lanes 1−4). Recombinantly expressed splicing factor 2 (SF2) was chosen as a positive control for interaction with p32 (Figure 2C, lane 5). (FLAG-tagged versions of the proteins were used for a sensitive detection on Western blots using MAb-FLAG; pUL97 lacking FLAG served as a specificity control [Figure 2C, lane 6].)

Eukaryotic translation factor 1δ (EF-1δ) is another cellular protein which is directly or indirectly phosphorylated by herpesviral protein kinases and especially UL13 exhibits a strong EF-1δ-phosphorylating activity.[5,8,20,21] Our *in vitro* kinase assays demonstrated for the first time a direct phosphorylation of EF-1δ by pUL97 (Figure 2D, lane 3). In addition, recombinantly expressed EF-1δ (Figure 2E, lanes 7, 9−10) as well as endogenous EF-1δ (data not shown) showed hyperphosphorylation upon coexpression of herpesviral protein kinases such as pUL97, UL13, and BGLF4. pR97 was negative (Figure 2E, lane 8), indicating that human EF-1δ was not recognized as a substrate of the RCMV-encoded kinase (note, that rat EF-1δ was not investigated). These data clearly underlined the functional similarities between the analyzed herpesviral protein kinases. UL13, however, as a member of the α-herpesviral protein kinases, seemed to possess greater functional distance toward pUL97 than β- and γ-herpesviral protein kinases. This point may be stressed by the fact that pUL97 and BGLF4 show another common activity (published previously), namely to interact with and phosphorylate the viral DNA polymerase processivity factor (pUL44 or BMRF1, respectively; Table 2). It is very likely that pR97 shows a similar phosphorylation activity since we were able to detect an interaction between pR97 and pR44 by coimmunoprecipitation analysis using lysates from RCMV-infected fibroblasts (data not shown). In contrast, HSV-1 UL13 seems not to bind and phosphorylate the DNA

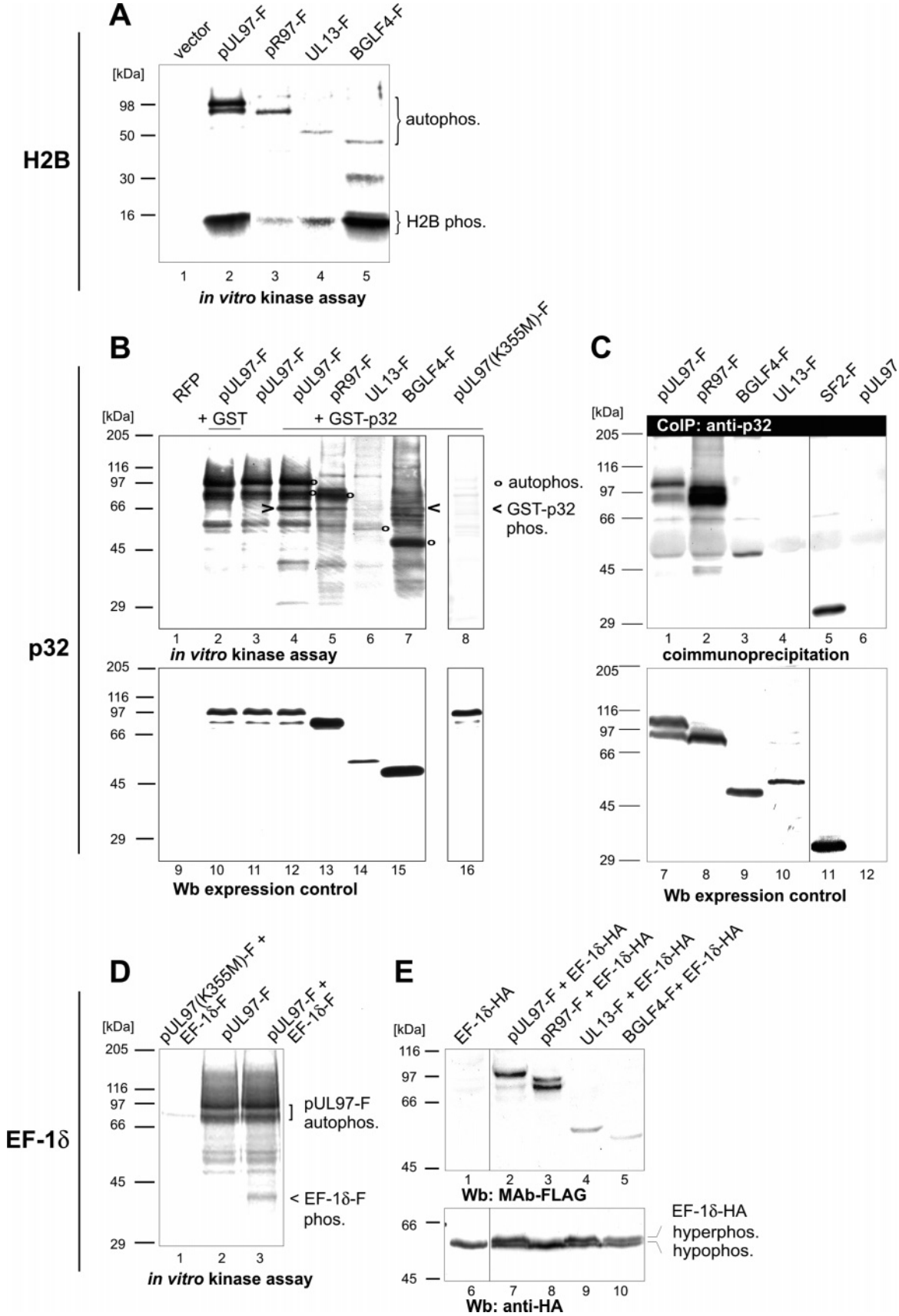

Figure 2. Phosphorylation activities and protein interactions of four herpesviral protein kinases. (A) HCMV pUL97, RCMV pR97, EBV BGLF4, and HSV-1 UL13 were produced in transfected 293 cells, immunoprecipitated with MAb-FLAG, and analyzed for *in vitro* kinase activity (H2B, purified histone 2B). (B) Phosphorylation of the cellular factor p32 by herpesviral protein kinases was investigated. (C) Direct interaction of endogenous cellular p32 with herpesviral protein kinases was analyzed by CoIP assay. (D) Phosphorylation of the cellular translation elongation factor EF-1*δ* by the pUL97 kinase was demonstrated by *in vitro* kinase assay. (E) Hyperphosphorylation of recombinantly expressed EF-1*δ* by herpesviral protein kinases in cotransfected cells was demonstrated by Western blot analysis. F, FLAG fusion tag; HA, hemagglutinin fusion tag; GST, gluthatione S transferase fusion protein; RFP, red fluorescent protein; MAb, monoclonal antibody; anti, polyclonal antiserum; Wb, Western blot.

Table 2. Summary of in Vitro Phosphorylation Activities of Four Herpesviral Protein Kinases

herpesvira l PKs	autophos[a]	human H2B[a]	humanp32 [b]	human EF-1d[c]	viral polymerase processivity factor	references
pUL97	+	+	+	+	+	1, 7, 46-47
pR97	+	+	+	−	n.d.	-
BGLF4	+	+	+	+	+	48−50
UL13	+	+	−	+	−	20−22

[a] Data are provided by the cited references and by Figure 2A,[a] Figure 2B,[b] or Figure 2D,[c] respectively.

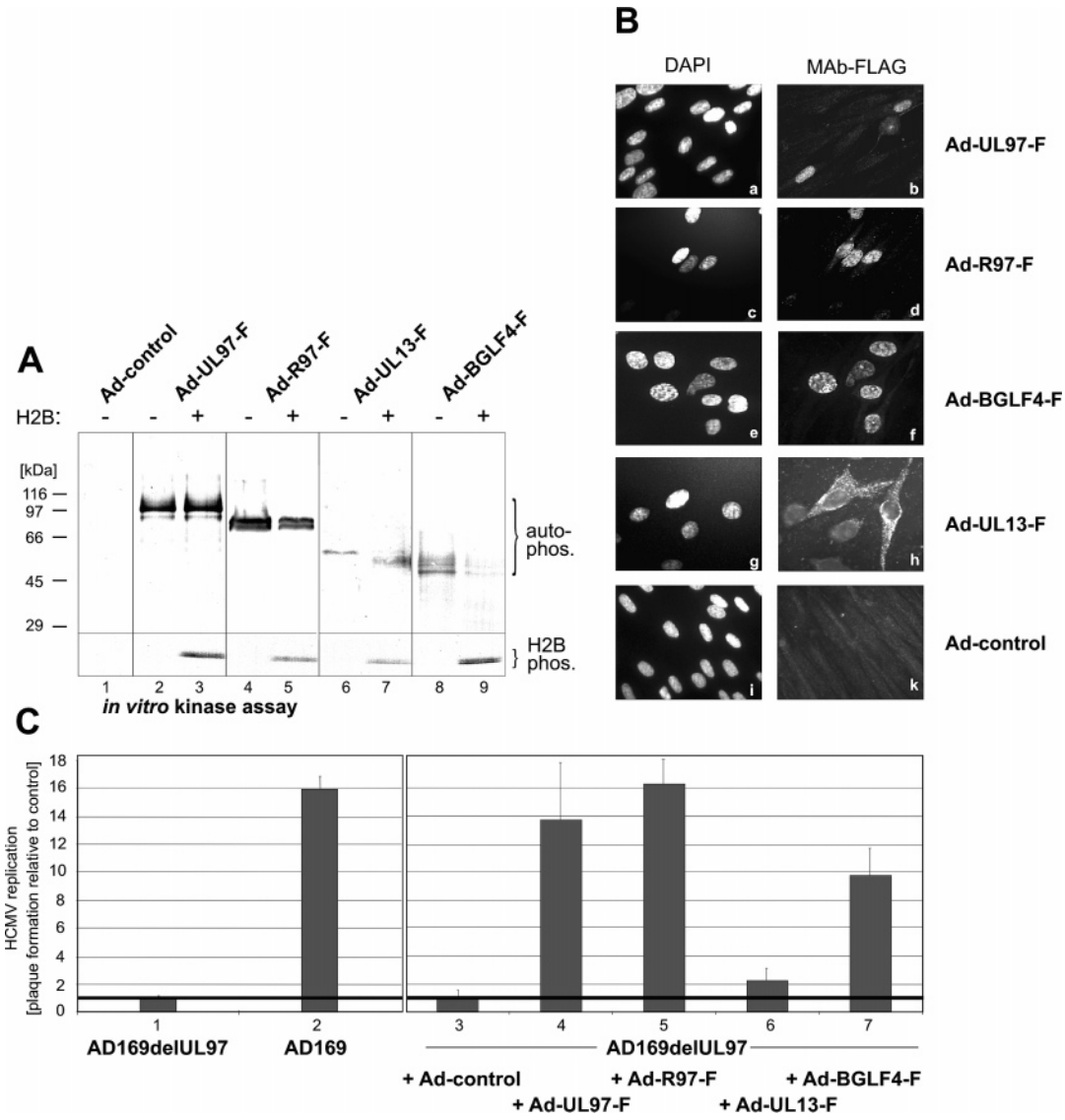

Figure 3. Trans-complementation assay on the basis of adenoviral (Ad) vector-mediated expression of herpesviral protein kinases in HCMV-infected cells (AD169delUL97 is a UL97-deleted mutant of HCMV strain AD169). (A) Recombinant Ad vectors were used for the expression of herpesviral protein kinases in 293 cells. At 2 days posttransduction, cells were lysed and used for an analysis of the activity of expressed kinases by the *in vitro* kinase assay. H2B, exogenously added histone 2B, was used as a phosphorylation substrate (15 μM). (B) HFFS were grown on cover slips, used for Ad transduction, and analyzed by indirect immunofluorescence after 2 days. Ad-control, empty vector. (C) HFFs were cultivated in a 12-well plate and analyzed by a trans-complementation assay. Transduction with Ad vectors was followed by infection with the UL97-deleted mutant of HCMV, AD169delUL97 (lanes 3−7); as a control, HCMV infection with the mutant (lane 1) or the parental strain AD169 (lane 2) was performed in the absence of Ad vectors. At 10 days postinfection, HCMV replication was quantified by plaque staining. All values were calculated as a mean of fourfold determinations (infections in duplicate and counting of plaques in duplicate).

polymerase processivity factor, UL42; instead, this activity may be displayed by the cellular kinase Cdk1 (cdc2).[22] Thus, as seen from data of the present study as well as published data, UL13 differed in activities which otherwise appeared to be conserved within herpesviral UL97 subfamily protein kinases.

Trans-Complementation of a UL97 Defect in HCMV Replication. Herpesviral protein kinases were transiently expressed in human cells by the use of recombinant adenovirus (Ad) vectors. This vector system admitted a high transduction efficiency and therefore a high level of recombinant expression in HCMV-permissive primary human fibroblasts (HFF). After Ad vector transduction, all kinases were expressed in an active form as demonstrated by autophosphorylation and H2B phosphorylation (Figure 3A). Interestingly, pUL97, pR97, and BGLF4 clearly localized in the nucleus, whereas UL13 mainly stained in form of a cytoplasmic signal (Figure 3B). Generally most herpesviral protein kinases, as described for their natural context of virus replication, show a nuclear accumulation, although they seem not to be restricted to this compartment. In particular UL13, which primarily localizes to the nucleus in

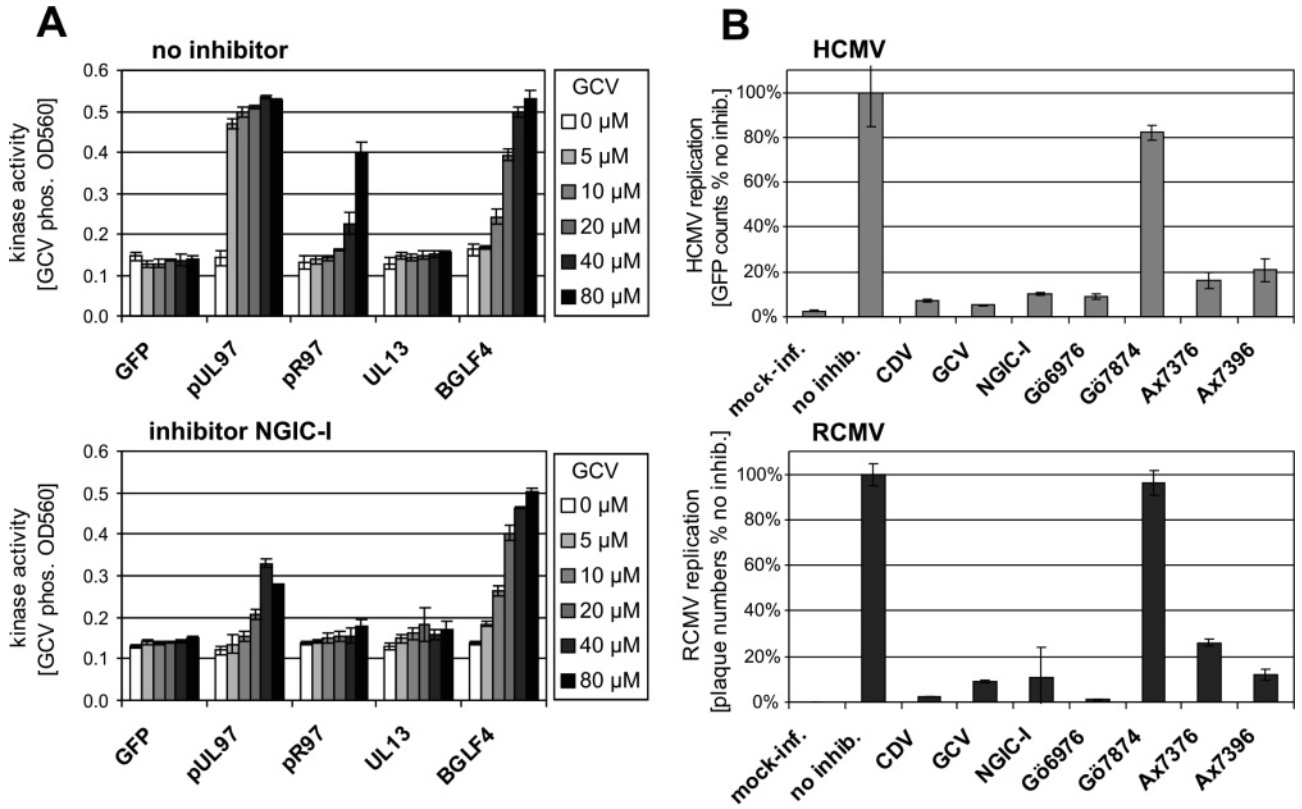

Figure 4. Determination of drug sensitivity. (A) Herpesviral protein kinases were recombinantly produced in 293 cells by transfection of expression plasmids. Ganciclovir (GCV) was added to the culture media as a nucleosidic phosphorylation substrate in a range of concentrations from 0 to 80 μM. An indolocarbazole-type protein kinase inhibitor (NGIC-I, 600 nM, with proven activity against pUL97[9,19]) was added to the culture media at the same time point as GCV (lower panel). (B) Primary human and rat fibroblasts, HFFs and REFs, were cultivated in 12-well plates and used for infection with HCMV AD169-GFP or RCMV strain Maastricht, respectively, or remained uninfected (mock-inf.). Viral replication occurred in the absence (no inhib.) or presence of antiviral drugs (CDV, cidofovir 1 μM; GCV, ganciclovir 20 μM; NGIC-I, Gö6976 and Gö7874, indolocarbazole-type protein kinase inhibitors 500 nM; Ax7376 and Ax7396, quinazoline-type protein kinase inhibitors 10 μM). At 7 days postinfection, viral replication was quantified as indicated. All values were calculated as a mean of fourfold determinations.

HSV-infected cells,[23] may additionally fulfill functions in the cytoplasm, such as the phosphorylation of EF-1δ.[20]

In order to assess the potential of these herpesviral protein kinases to complement a kinase defect in the HCMV genome, we established a quantitative trans-complementation system. For this, HFFs were transduced with the constructs for expression of each one of the herpesviral protein kinases and subsequently infected with a mutant of HCMV carrying a deletion in the UL97 coding region (AD169delUL97).[12] AD169delUL97 virus showed a marked reduction in replication efficiency compared to the parental virus (Figure 3C, lanes 1 and 2). This reduction could be complemented in trans by pUL97 as well as pR97, and to a lesser extent (app. 60%) also by BGLF4 (Figure 3C, lanes 4, 5, and 7), but not significantly by UL13 (lane 6). Thus, considering the *in vitro* situation of HCMV replication, pUL97 function seemed to be more related to pR97 and BGLF4 than to UL13.

Sensitivity of Herpesviral Protein Kinases toward Kinase Inhibitors. Herpesviral protein kinases were expressed in 293 cells and analyzed by the use of kinase in-cell-activity assays. For this, cells expressing herpesviral protein kinases were treated with increasing concentrations of ganciclovir (GCV), which here served as an indicator substance of kinase activity: specific phosphorylation of GCV resulted in an induction of cytotoxic signals which could be quantified with this assay (Figure 4A, upper panel). As a clear result, pUL97, pR97, and BGLF4 were positive for GCV phosphorylation, while UL13 was negative (for details of the assay, including controls such as pUL97-[K355M] see ref 19). From a quantitative view, pUL97 was most efficient concerning this activity, BGLF4 was intermediate and pR97 was comparatively low. Equivalent expression levels of the kinases in transfected cells were controlled by Western blot analysis (data not shown). Importantly, the activity of pUL97 and pR97 could be blocked by the addition of a known inhibitor of pUL97 belonging to the indolocarbazole class (NGIC-I,[19] Figure 4A, lower panel). BGLF4 was not sensitive to NGIC-I, as published before.[9] Thus, the analysis revealed a close similarity between pUL97 and pR97 concerning GCV phosphorylation and NGIC-I-mediated inhibition of this activity.

This similarity prompted us to investigate the underlying structural features by molecular modeling of pUL97 and pR97. Generally, the amino acids forming the inhibitor binding pocket for indolocarbazoles (such as staurosporine [STP], NGIC-I, Gö6976, and others; Figure 1E) is highly conserved among Ser/Thr protein kinases. There are, however, also several residues, which are conserved only in a subset of kinases and may therefore explain differences in the sensitivity toward inhibitors. The contacts of this group of residues were analyzed in the crystal structure of STP-bound PKC θ (Figure 1F) and in the modeled complexes of pUL97 (Figure 1G) and pR97 (Figure 1H) bound to NGIC-I. This analysis revealed that the level of sequence conservation in the ligand binding pocket was significantly higher between pUL97 and pR97 compared to PKC θ. In particular those three amino acids which contacted the nonglycosidic moiety of NGIC-I were highly conserved between pUL97 (M460, F419, C480) and pR97 (M365, Y324, C385) which was consistent with the measured similarity of inhibitor sensitivity.

On the basis of virus replication, human (HCMV) and rat cytomegaloviruses (RCMV) also showed a very similar sensitivity toward protein kinase inhibitors (Figure 4B). It is known that specific indolocarbazoles and quinazolines exhibit strong and quite selective inhibitory activity both toward HCMV pUL97 kinase activity and HCMV replication.[9,24] Here we used three indolocarbazoles (NGIC-I and Gö6976, possessing pUL97-inhibitory activity; Gö7874, lacking this activity), two quinazolines (Ax7376 and Ax7396, both pUL97 inhibitors) and two pharmacological inhibitors of viral DNA synthesis (GCV, antiviral activity dependent on pUL97-mediated phosphorylation; CDV, pUL97-independent). As a striking result, the sensitivity profile, regarding these selected inhibitors, was almost identical for HCMV (Figure 4B, upper panel) and RCMV (lower panel). Particularly the efficacy of pUL97-specific protein kinase inhibitors is similarly high for HCMV and RCMV. This suggested that pUL97 and pR97 possess a similarly high importance for viral replication. Moreover, as these compounds were considered as promising candidates of novel anti-HCMV drugs, experimental infection with RCMV (and possibly related animal cytomegloviruses) may provide a suitable animal model for analyzing the efficacy of pUL97 protein kinase inhibitors *in vivo*.

Postulated Functional Roles of Herpesviral UL97 Subfamily Protein Kinases. The functional roles of herpesviral protein kinases are not completely understood. This may be based on a number of relatively complex protein interactions and phosphorylation activities. One important aspect of complexity is the low level of sequence conservation. This points to a specialization of these protein kinases within the replication cycles of the individual herpesviruses. It is striking that α-herpesviruses code for at least two protein kinases, i.e., members of the UL97 and US3 subfamilies, while *β*- and *γ*-herpesviruses code for only one protein kinase of the UL97 subfamily. Interestingly, the increasing amount of published functional data raises the suggestion that those functions fulfilled separately by either of the two α-herpesviral protein kinases may be unified in the case of one single *β*-/*γ*-herpesviral protein kinase. An example is given by the activities of HSV-1 UL13, i.e., the phosphorylation of viral and cellular regulatory proteins (e.g., RNAP II and EF-1*δ*[20,25]), which are distinct from those of HSV-1 US3, i.e., the involvement in redistribution of the nuclear lamina.[26] For HCMV pUL97, on the other hand, an involvement in all of these functions was reported, possibly indicating that the importance of pUL97 for viral replication may be superordinate to that of its homologue UL13. In fact, it has been shown that UL13 is dispensable for HSV-1 replication *in vitro*,[11] while the deletion of UL97 in the HCMV genome leads to a severe replication defect.[2,6−7,12]

This hypothesis is further illustrated by the fact that the capacity of functional complementation of herpesviral protein kinases may be dependent on the background of the analyzed viral genome. As shown in this study (Figure 3C), recombinant UL13 was not able to complement a UL97 defect in the HCMV genome and this seemed to be in conflict with a previous report by Ng et al.[27] The authors used a UL13 deletion mutant of HSV-1 and demonstrated a functional complementation by pUL97. These controversial aspects may be explained by two points. First, the interaction with viral kinase-accessory proteins might be more important for UL13 than for pUL97, which may correspond to a complementation in the HSV-1 but not in the HCMV context. In fact, a functional linkage of UL13 with other HSV-1 regulatory proteins was reported.[25,28] Second, pUL97 might provide a greater number of functions than UL13 and apparantly possesses higher functional importance for HCMV replication than UL13 for HSV-1. This assumption was supported by the pattern of activities described in Figure 2 and Table 2. Particularly the interaction with the cellular factor p32 was lacking for UL13, but was conserved among those kinases showing full or partial complementation of a pUL97 defect. Therefore, the concept that pUL97 comprises many functions and that some of these are not covered by α-herpesviral homologues is strengthened by our findings.

pUL97 and Related Kinases as Novel Anti-Herpesviral Drug Targets. pUL97 has been validated as an interesting drug target in the course of the development of novel anti-herpesviral drugs, and those studies underlined a high functional importance of the kinase for viral replication.[3,19,24] Protein kinase inhibitors of the chemical classes benzimidazoles, indolocarbazoles, and quinazolines were described possessing strong inhibitory potential toward both the pUL97 kinase activity and HCMV replication *in vitro*. Further improvements in efficacy and selectivity of such drugs may be accelerated by the optimization of structural kinase models and computer-based medicinal drug chemistry. An example of such future prospects is provided by indolocarbazoles which act as competitive inhibitors at the ATP binding site. Some derivatives possess clear selectivity to ATP binding sites of cellular protein kinases such as PKC,[29] while others appear to be specifically directed to herpesviral kinases such as pUL97.[9,19,30] The relatively high degree of remaining divergence in the structural features found for cellular compared to herpesviral protein kinases, i.e., those regions spacing between the highly conserved motifs of the kinase domains (Figure 1), may open opportunities for an optimized drug design. In order to address this point in more detail by the present study, the protein−inhibitor contacts were compared between a crystal structure of STP-bound PKC,[31] and models of NGIC-I bound to pUL97 or pR97. Particular attention was paid to those residues of PKC that presumably contact the ligand, but are not conserved in the viral kinases (Figure 1E). There was a total of nine "nonconserved" residues which contact STP in complex with PKC (Figure 1F). Analysis of the equivalent sequence positions in pUL97 (Figure 1G) and pR97 (Figure 1H) showed that the side-chain properties of the respective amino acids differed significantly compared to the cellular kinase, but were moderately conserved between the two viral kinases. These residues might represent candidates for the design of more specific drugs in the future.

The availability of these models also allowed us to address the structural basis for the high sensitivity of pUL97 and pR97 for the inhibitor NGIC-I. There were three residues of pUL97 (M460, F419, C480) which were very likely to contact the nonglycosidic part of NGIC-I representing the structural difference between NGIC-I and STP (Figure 1G). Two of these residues were strictly conserved between pUL97 and pR97 and F419 was replaced by the highly similar Y324 (Figure 1H). The high sensitivity of the two herpesviral protein kinases for NGIC-I compared to a low sensitivity to STP could most likely be attributed to the presence of an aromatic amino acid in pUL97 (F419) and pR97 (Y324), while a smaller and polar residue (D465) was present at the respective sequence position in PKC (Figure 1F−H). D465 might be sufficiently small to allow the binding of STP in PKC, but modeling suggested that the bulkier aromatic side-chain of F419/Y324 led to clashes with the glycosidic moiety of STP thereby decreasing the affinity for this inhibitor. On the contrary, the flexible side-chain of NGIC-I could be accommodated even in the presence of the aromatic residue in pUL97 and pR97. Therefore, a selective binding of

pUL97 and pR97 to indolocarbazole NGIC-I seemed favorable. Although M460 in pUL97 was expected to form only backbone contacts to NGIC-I, replacement of this residue by isoleucine was demonstrated previously to increase the sensitivity of NGIC-I binding.[9] On the basis of the present model, this effect can most likely be explained by additional hydrophobic side-chain contacts between I460 and the methylene groups of the NGIC-I side-chain.

Conclusions

In the present SAR study performed with pUL97, pR97, BGLF4, and UL13, we provide data for the following main conclusions: (i) overall sequence homology is low but the computational approach employing fold recognition techniques clearly revealed structural similarity to Cdk2, (ii) a three-dimensional pUL97 model suggests a high level of conservation of the functionally important residues in ATP binding sites and the catalytic center, (iii) an analysis of *in vitro* activities of the herpesviral protein kinases (i.e., patterns of phosphorylation and interaction with cellular proteins) showed marked parallels for pUL97, pR97, and BGLF4, whereas UL13 behaved differently, (iv) trans-complementation experiments demonstrated that pUL97, pR97 and partially BGLF4, but not UL13, were able to complement a UL97 defect in the HCMV genome, and (v) the sensitivity toward protein kinase inhibitors showed strong similarities for the cytomegaloviral kinases pUL97 and pR97, both on the levels of kinase activity as well as viral replication. Combined, the findings demonstrate that UL97 subfamily of herpesviral protein kinases share several but not all of their complex functional properties and a particularly close similarity is suggestive for pUL97 and pR97 (e.g., autophosphorylation, phosphorylation of similar substrates, functional complementation and inhibition by specific protein kinase inhibitors). Thus, on the basis of marked parallels both in structural features as well as enzymatic activities, future SAR investigations may help to determine the fine-structure of kinase domains and may thereby promote a novel approach in anti-herpesviral drug design.

Methods

Computer-Based Prediction of Three-Dimensional Protein Structures. Sequence analysis and structure prediction of herpesviral protein kinases, such as human cytomegalovirus (HCMV) UL97,[32] rat cytomegalovirus (RCMV) R97,[33] Epstein−Barr virus (EBV) BGLF4,[34] and herpes simplex virus type 1 (HSV-1) UL13,[35] was performed using Structure Prediction Meta Server (http://bioinfo.pl/ meta) and yielded the modeling template PDB 1vyw chain C (human Cdk2).[17] Structural models of the herpesviral protein kinase domains based on Cdk2 were built in ESyPred3D.[36] Model evaluations were performed by ProQ,[37] with reference to secondary structure predictions by PSIPRED.[38] Inhibitor binding was analyzed with LigPlot,[39] using the staurosporine (STP)-bound form of PKC θ as a reference.[31]

Plasmid Constructs and Recombinant Adenoviruses. pcDNA-UL97, pcDNA-UL97-FLAG, pUL97(K355M)-FLAG, pcDNA-BGLF4-FLAG, and pcDNA-SF2-FLAG were described before.[7,19] pME-EF-1δ-FLAG and pGEM-EF-1δ were kindly provided by Y. Kawaguchi (Nagoya Univ. School Med., Japan). Further expression plasmids were generated by the insertion of PCR products into vectors pcDNA3.1 (Invitrogen), pACT (Promega) and pPM7tet (transfer vector for Ad recombination; kindly provided by Matt Cotten, Axxima Pharmaceuticals). PCR products for ORFs R97 and UL13 were obtained by the use of total DNA from virus-infected cells, i.e., RCMV strain Maastricht-infected rat fibroblasts or HSV-1 clinical isolate-infected Vero cells. PCR was performed using Vent DNA polymerase (New England BioLabs) in 35 cycles (denaturation 40 s at 95 °C, annealing 40 s at 50 °C, and polymerization 120 s at 72 °C). Oligonucleotide primers for PCR were purchased from Biomers; gene-specific/coding sequences are underlined, restriction sites in boldface and FLAG sequence in italics:

5-R97-*Eco*RI,
TGA**GAATTC***ATGGAGAACACGCCGCCCCCG*;
3-R97-FLAG-*Xba*I,
T G A **T C T A G A** *C T A C T T G T C G T - CATCGTCTTTGTAGTCGGGGAACAGGGAGA AGGGGCCG*;
5-UL13-*Eco*RI,
TGA**GAATTC***ATGGATGAGTCCCGCAGACA*;
3-UL13-FLAG-*Xba*I
T G A **T C T A G A** *C T A C T T G T C G T - CATCGTCTTTGTAGTCCGACAGCGCGTGCC GCGCGC*;
5-EF1d-*Sal*I,
TAG**GTCGAC**TT*ATGGCTACAAACTTCCTAGCACATG*;
3-EF1d-HA-*Xba*I,
T A G **T C T A G A** *C T A A G C G T A A T C T G G A A - CATCGTATGGGTAGATCTTGTTGAA AGCTGCGATATCG*;
5′-UL97-*Pac*I,
TGA**TTAATTAA***ATGTCCTCCGCACTTCGGTC*;
HSV1-UL13-fwd-*Pac*I;
TGA**TTAATTAA***ATGGATGAGTCCCGCAGAC*;
EBV-BGLF4-fwd-*Pac*I,
TGA**TTAATTAA***ATGGATGTGAATATGGCTG*;
R97-fwd-*Pac*I,
TGA**TTAATTAA***ATGGATAACACTCCTCCCC*;
FLAG-*Kpn*I,
TGA**GGTACC***TTACTTGTCGTCATCGTCTTT*;
FLAG-*Eco*RV,
TGA**GATATCTTA***CTTGTCGTCATCGTCTTTG*.

Recombinant adenoviruses (Ad) were constructed on the basis of the replication-defective vector pAdtetKK (Ad type 5 with deletions in genomic regions E1 and E3; kindly provided by Matt Cotten, Axxima Pharmaceuticals).[40,41] Plasmid DNA of pAdtetKK was linearized with *Pac*I, dephosphorylated with shrimp alkaline phosphatase (Fermentas), and purified by the use of the QIAquick Gel Extraction Kit (Qiagen). Fragments for recombination were excised from the transfer constructs by restriction digest with *Bam*HI (pPM7tet-R97-FLAG and pPM7tet-UL13-FLAG) or *Nde*I (pPM7tet-UL97-FLAG and pPM7tet-BGLF4-FLAG), respectively, and purified via agarose gel separation. For the recombination, linearized vector and one of the recombination fragments (1:5 v/ v) were transformed into *E. coli* BJ5183. Clones were selected on ampicillin plates, used for the isolation of adenoviral DNA, and analyzed by restriction digest and PCR. Positive clones were used for the reconstitution of recombinant Ad by polyethylenimine transfection into 293 cells. Approximately 14 days posttransfection, cells and supernatants were harvested, lysed by freezing/thawing/vortexing, and used as an inoculum for a second round of Ad growth on 293 cells to amplify viral titers. After complete cell lysis (approx. 2−3 days) culture media were collected, separated from cell debris by centrifugation, and used to purify viral preparations by ultracentrifugation (34 000 rpm, 1 h, 4 °C) through 36% sucrose cusions. Pellets were resuspended in TNCA buffer (100 mM NaCl, 10 mM $CaCl_2$, 0.02% NaN_3, 50 mM Tris-HCl pH 8.0) and stored as Ad stocks at −80 °C.

Cell Culture, Viruses, and Transfections. Primary human foreskin fibroblasts (HFFs) and primary rat embryonic fibroblasts (REFs) were cultivated in MEM, 293 cells in DMEM containing 5% fetal bovine serum. Human cytomegaloviruses AD169, AD169-GFP, and AD169delUL97 were propagated in HFFs, rat cytomegalovirus (RCMV) strain Maastricht in REFs. Viral replication was quantified by plaque assay or GFP fluorometry. According to the low efficiency of replication, the titer of mutant AD169delUL97 was determined at 28 days postinfection while AD169, AD197-GFP, and RCMV were determined at 7 days postinfection. Transient transfection of 293 cells was performed at a cell confluency of 75% by the use of Lipofectamin Plus according to the protocol of the manufacturer (Invitrogen) or by the polyethylenimine transfection

method (Schregel et al., 2006. Mapping of a self-interaction domain of the cytomegaloviral protein kinase pUL97. *J. Gen. Virol.*, submitted)

Coimmunprecipitation Assay and Western Blot Analysis. 293 cells ($\sim 5 \times 10^6$) transfected with expression plasmids or transduced with recombinant Ad vectors were lysed in 1 mL of CoIP buffer (50 mM Tris-HCl pH 8.0, 150 nM NaCl, 5 mM EDTA, 0.5% NP-40, 1 mM PMSF, 2 μg/mL aprotinin, 2 μg/mL leupeptin and 2 μg/mL pepstatin) and used for coimmunoprecipitation (CoIP) with 4 μL of anti-p32 polyclonal antiserum (kindly provided by W.C. Russel, U.K.) for 2 h at 4 °C under rotation. Protein A-sepharose beads were added to the CoIP reactions (2.5 mg, 2 h at 4 °C, Amersham Pharmacia Biotech) before complexes were pelleted, washed, and used for separation in 12.5% SDS-PAGE followed by Western blot detection of FLAG-tagged proteins with MAb-FLAG (M2, Sigma). For Western blot analyses, protein samples were denatured under reducing conditions and SDS-PAGE was performed according to standard protocols. Proteins were detected on Western blots using anti-HA.11 polyclonal antiserum (Babco) or MAb-FLAG and peroxidase-conjugated secondary antibodies (Dianova). Signals were developed in an enhanced chemiluminescence reaction (ECL Western Detection Kit; Amersham Pharmacia) according to the manufacturer's instructions.

***In Vitro* Kinase Assay (Protein Phosphorylation).** Autophosphorylation and substrate phosphorylation was determined *in vitro* after immunoprecipitation of herpesviral protein kinases from transfected 293 cells as described.[9] Putative phosphorylation substrates were either produced by cotransfection (EF-1δ-F) and immunoprecipitated together with one of the analyzed protein kinases or added exogeneously as purified proteins (GST-p32,[7] histone 2B, Roche). Phosphorylation reactions were performed in the presence of 2.5 μCi of [γ-^{33}P]ATP. Phosphorylated products were separated in a 12.5% SDS-PAGE and signals were detected by exposure on autoradiography films or phosphoimager plates.

Kinase in-Cell-Activity Assay (GCV Phosphorylation). This screening method for inhibitors of pUL97 kinase activity was performed as described previously.[7,19] In brief, 293 cells were grown in 96-well plates, transfected with a kinase expression plasmid or a control plasmid, and incubated with medium containing serial concentrations of ganciclovir (GCV). Of note, in this procedure GCV does not act as an inhibitor but as an indicator of GCV phosphorylation by inducing a quantifiable signal (i.e., cytotoxicity). Upon phosphorylation of GCV in cells recombinantly expressing pUL97 or another GCV-recognizing herpesviral protein kinase, but not in cells with a control plasmid, cytotoxicity was induced which could be quantified in terms of a color conversion (yellow to red) of the phenol red-containing culture medium at a wavelength of 560 nm. The addition of inhibitors of the pUL97 kinase activity (e.g., NGIC-I) to the culture medium immediately after transfection suppressed this cytotoxic effect.

Trans-Complementation Assay. HFFs were cultivated in 12-well plates (125 000 cells per well) and transduced with a 500-μL inoculum volume of recombinant Ad constructs for 4 h at 37 °C. Inoculi were adjusted according to the expression levels of the recombinant proteins determined by Western blot and immunofluorescence analysis (MOI 100 of Ad-control construct used as a basis for adjustment). One day posttransduction, cells were infected with HCMV AD169delUL97 or AD169 (MOI 0.1) and overlaid with 0.6% agarose medium. After a cultivation period of 10 days, plaques were stained with 1% crystal violet and counted under the microscope.

Indirect Immunofluorescence Assay. Primary human foreskin fibroblasts (HFF) were grown on coverslips for transduction with recombinant Ad constructs. 2 days posttransduction, cells were fixed with ice-cold methanol for 10 min and blocked by preincubation with nonspecific horse serum for 30 min at 37 °C. MAb-FLAG was incubated as the primary antibody for 90 min at 37 °C, and secondary antibody anti-mouse-FITC (Dianova) was incubated for 45 min at 37 °C (nuclear counterstaining with DAPI Vectashield mounting medium; Vector Laboratories). Data for immunofluorescence were collected by the use of an Axiovert-135 microscope at magnifications of 400× and 630× (Zeiss). Images were recorded with a Cooled Spot Color Digital Camera (Diagnostic Instruments).

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