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A cell free protein fragment complementation assay for monitoring the core interaction of the human cytomegalovirus nuclear egress complex

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ABSTRACT

Certain viral protein–protein interactions provide attractive targets for antiviral drug development. Recently, we described a β-lactamase based protein fragment complementation assay (PCA) to study the core interaction of the nuclear egress complex (NEC) of different herpesviruses in cells. Now, to have a cell free assay for inhibitor screens, we expressed split β-lactamase tagged interaction domains of the viral pUL50 and pUL53 proteins representing the NEC of human cytomegalovirus (HCMV) in bacteria. After validation and basic characterization of this NEC–PCA, we tested peptide inhibitors of the pUL50–pUL53 complex. We show that peptides resembling sequences of the first conserved region of pUL53 can inhibit the NEC–PCA. This, on one hand, indicated that the core interaction in the HCMV NEC is mediated by a linear motif. On the other hand it proved that this new pUL50–pUL53 interaction assay allows a simple cell free test for small molecular inhibitors.

1. Introduction

The human cytomegalovirus (HCMV) is a leading cause of congenital infection and of complications in immunocompromised patients. HCMV infections are primarily controlled by antiviral therapies targeting the viral DNA replication, and these are accompanied with serious side effects (De Clercq and Holy, 2005). In the past decade a few new viral targets and inhibitors have been identified providing potential alternatives for anti-HCMV therapeutics (Goldner et al., 2011; Hwang et al., 2007; Krosky et al., 2003; Loregian and Coen, 2006; Nixon and McVoy, 2004).

Most licensed antiviral drugs target viral enzymatic functions. Tests for these activities are well established *in vitro* and therefore more amendable for screening projects. However, targeting herpesviral protein–protein interactions (PPIs), which represent the structural basis of all essential viral functions became an attractive alternative approach for antiviral drug development (Loregian and Coen, 2006; Loregian and Palu, 2005). Even peptide inhibitors of specific viral PPIs of key cytomegalovirus function were described and identified using classical approaches (Loregian et al., 2003).

Cytomegaloviruses share the architecture of their virion with all herpesviruses. Furthermore, the main pathways during virus replication are conserved, which is reflected by a core set of conserved genes (Davison, 2010; Mocarski, 2007). Notably, the morphogenesis of infectious herpesvirus particles is characterized mainly by conserved PPIs (Fossum et al., 2009). One conserved interaction is formed by the pUL34 and pUL31 protein families, which are together with several other cellular and viral factors components of the nuclear egress complex (NEC) (Fossum et al., 2009; Johnson and Baines, 2011; Milbradt et al., 2009; Schnee et al., 2006). The core complex of the NEC is found at the inner nuclear membrane and is crucial for the primary envelopment of all studied herpesviruses (reviewed in Johnson and Baines, 2011; Mettenleiter, 2004). It was shown that the NEC is involved in the destabilization of the nuclear lamina (Bjerke and Roller, 2006; Gonnella et al., 2005; Klupp et al., 2007; Muranyi et al., 2002; Reynolds et al., 2004), reorganization of viral and cellular intranuclear structures (Simpson-Holley et al., 2004, 2005), and is supposed to act as a docking station for DNA filled capsids (Klupp et al., 2006; Popa et al., 2010; Yang and Baines, 2011). For CMVs, the interaction of murine CMV (MCMV) pM50 and pM53, the members of the pUL34 and pUL31 protein families, respectively, was characterized first and shown to be essential for productive infection (Bubeck et al., 2004; Muranyi et al., 2002). The transmembrane protein pM50 and pM53 with nuclear localization, form a non-obligatory interaction at the inner nuclear membrane (Bubeck et al., 2004; Lotzerich et al., 2006). Mutagenesis studies on pM53 furthermore identified the minimal binding site for pM50 to be in the first conserved region (CR1) (Lotzerich et al., 2006). Studies on the HCMV homologs, pUL50 and pUL53, partially confirmed the above findings (Milbradt et al., 2007; Sam et al., 2009; Schnee et al., 2006).

Abbreviations: HCMV, human cytomegalovirus; NEC, nuclear egress complex; PPI, Protein–protein interactions; CR1-4, conserved region one to four; Bla, β-lactamase; PCA, protein fragment complementation assay.

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Moreover, the *in vitro* reconstitution of the NEC of HCMV provided the first cell free model to characterize this essential viral PPI in molecular detail (Sam et al., 2009).

The interaction of NEC proteins from all herpesvirus subfamilies was monitored and characterized by a protein fragment complementation assay (PCA) (Schnee et al., 2006). This cell based PCA was established by fusion of the N- and C-terminal split-parts of the TEM-1 β-lactamase (Bla) of *Escherichia coli* (Galarneau et al., 2002) to the respective NEC proteins and applied in transfection experiments in cell culture. In contrast to other methods for monitoring PPIs, such as the yeast-two-hybrid system (Y2H), co-immunoprecipitation, FRET/BRET and bimolecular fluorescence complementation (BiFC), the Bla based PCA is reversible and applicable for proteins independent of their intracellular localization or biochemical features (reviewed in (Michnick et al., 2007)). It can be performed in different cell types of eukaryotic and prokaryotic origin, without obligatory destruction of the cells and it is not dependent on special laboratory equipment. PCAs attracted interest for mapping biochemical pathways and as a tool for genomic networking. Further, PCAs were suggested as tool for the validation of drugs and cell based high-throughput screens for inhibitory compounds (Luker et al., 2004). However, beside the pioneering work showing that BiFC is feasible *in vitro* (Hashimoto et al., 2009), no cell free PCA utilizing reconstitution of reversible enzyme activity was described for PPIs.

Here we show a new application of the NEC–PCA: Using fusion proteins expressed in bacteria we established an *in vitro* assay (iPCA) for the core interaction of the HCMV NEC. After validation and basic characterization of this iPCA, it was used to test synthetic peptides for their inhibitory potential on the essential NEC interaction mediated by the conserved region (CR) of pUL50 and CR1 of pUL53. We thereby established a new platform for screening of inhibitors of the HCMV–NEC interaction and limited the residues, which were sufficient for binding pUL50 to a linear motif derived from pUL53.

2. Materials and methods

2.1. Synthetic peptides and oligonucleotides

Peptides C1 (aa63–85 of pUL53): DLHDIFREHPELELKYLNMMK-MA, C2 (aa63–92 of pUL53): DLHDIFREHPELELKYLNMMKMAITGK-ESI, C3 (aa86–92 of UL53): itgkesi and C4 (aa72–92 of UL53): Pelelkylnmmkmaitgkesi and oligonucleotides were purchased from Metabion GmbH. The sequences of oligonucleotide primers used in this study are listed in the Supplementary Table S1.

2.2. Plasmids construction

For bacterial expression of the pUL50 and pUL53 fragments and Bla fusions thereof the respective coding sequences were amplified by PCR and cloned into the pET vector system (Novagen). For the design of a well-defined interacting unit, pUL50 and fragments of pUL53 were co-expressed from a bicistronic construct and pUL50 was C-terminally His-tagged. The Bla fragments were fused to the N-terminus of the viral coding sequences and fusion constructs were further labeled by a His-tag for purification. For detection and quantification, the pUL53 and pUL50 Bla fusions were N-terminally tagged by an HA-tag, fragments of pUL53, pUL31 and a pUL53/pUL31 fusion by a Flag-tag. (see details of plasmid construction in Supplementary method S1 and a comprehensive table describing the details of all construct is provided in Supplementary Table S1).

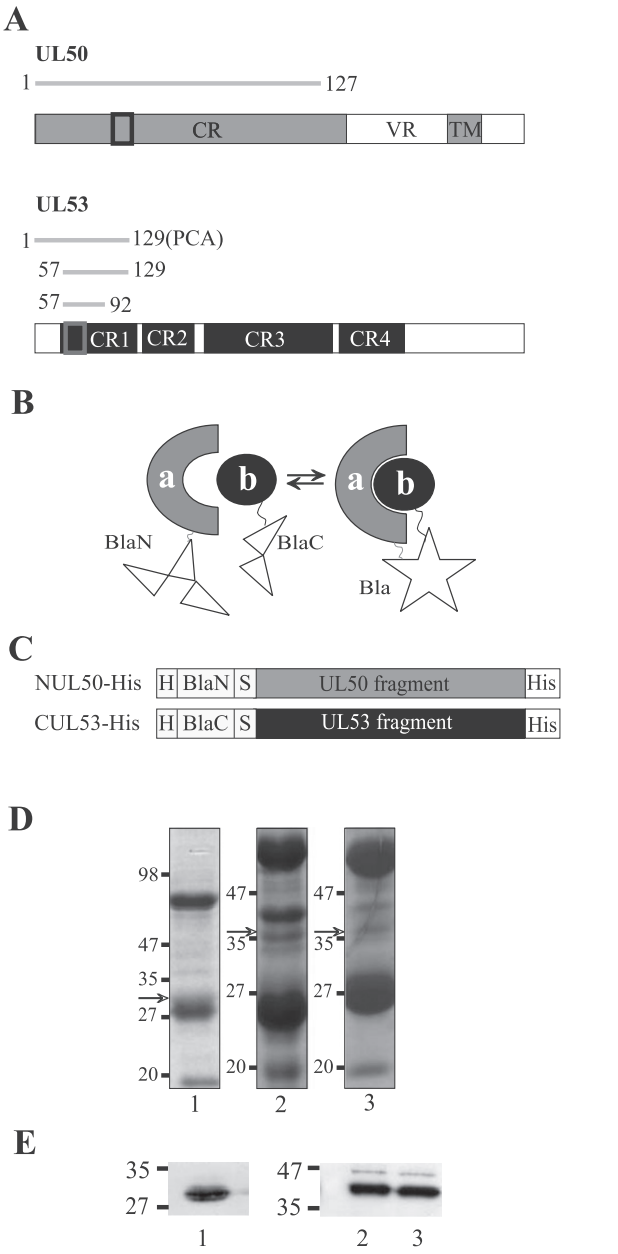

Fig. 1. Design and purification of the β-lactamase fusion protein of pUL50 and pUL53 fragments for iPCA (A) Schematic representation of pUL50 and pUL53 of HCMV. pUL50 contains an N-terminal conserved region (CR) and a predicted transmembrane helix near to its C-terminus (TM) separated by a variable region (VR). pUL53 consists of four conserved regions (CR1-4). The predicted binding sites are located near to the N-termini of both proteins (open black and grey boxes). The fragments which were used in this study are indicated with gray lines aligned to the representation of pUL50 (aa1–172) or to the pUL53 (aa1–129 fragment used in the PCA, and aa57–92 (UL53_1) or aa57–129 (UL53_2) fragments used in the co-purification experiment). (B) Simplified scheme of a protein fragment complementation assay (PCA). The N- and C-terminal part of a reporter enzyme (white), here the TEM-1 β-lactamase (Bla), are fused to two interacting proteins (a and b). If the proteins a and b interact, the proximity of the enzyme fragments BlaN and BlaC allows the folding and reconstitution of the active enzyme (star). (C) Schematic representation of the N-terminally HA (H)- and C-terminally His-tagged constructs. The N-terminal part of Bla, representing the residues 24–194 (N, BlaN), and the C-terminal part (C, BlaC), representing the Bla residues 196–286, were fused to fragments of the HCMV proteins pUL50 (wild type (wt) or a mutant) and pUL53, linked by a glycine/serine spacer (S). (D/E) Elutions of the Ni^{2+}–NTA purification of CUL53-His (1) NUL50-His (2) NUL50DM-His (3) were analyzed by separation of the indicated samples on a 12% SDS–PAGE and Coomassie–blue staining (D) and by an HA-specific Western Blot (E). Numbers on the left of the panels indicate the position and size of the protein marker in kDa. The constructs were detected at the predicted size about 42 kDa (NUL50-His and NUL50DM-His) and 28 kDa (CUL53-His). Arrows indicate the respective protein bands on the gel.

2.3. Expression of recombinant proteins in E. coli

BL21 (DE3) RIL cells (Stratagene) were transformed with constructs in the pET24 or pET28 background and grown at 37 °C to an OD_{600} of 0.6. Expression was induced by 0.5 mM IPTG for 16 h at 20 °C. Cells were harvested by centrifugation, resuspended in buffer A (50 mM Tris (pH 8.0), 300 mM NaCl, 10 mM β-mercaptoethanol) and lysed by sonication. For all recombinant proteins the expression and cell lysis was performed as described above, except for UL53(57–84) and CR1UL31, which were expressed 4.5 h at 37 °C. After sonication and centrifugation, the supernatant was supplemented by protease inhibitors (Roche) diluted to 12 μg/μl of total protein and normalized for the content of Flag-tagged polypeptides determined by an anti-Flag-ELISA. Fifty microliter of Anti-Flag M2 agarose (Sigma) was added to 1 ml of protein lysates and incubated for 4.5 h at 4 °C. The agarose was washed 5 times with 1 ml of Anti-Flag M2 agarose wash buffer (Sigma) and once with Na–phosphate buffer (0.1 M, pH 7.0).

2.4. Purification of the pUL50/pUL53 complex and Bla-fusion proteins

After centrifugation of sonicated protein samples, the supernatant was loaded onto a Ni^{2+}–NTA (Qiagen) column with 600 μl bed volume, equilibrated with buffer A. After washing with 30 ml of buffer A and 4 ml of buffer A containing 5 mM imidazole, bound protein was eluted with buffer A containing 250 mM imidazole. Bla-fusion proteins were not further purified and stored in 25% glycerol at –80 °C. The pUL50/pUL53 protein complex was purified further by anion-exchange chromatography (MonoQ, GE Healthcare). The column was equilibrated with buffer B (50 mM Tris (pH 9.0), 1 mM EDTA, 5 mM DTT) and the complex was eluted with a continuous gradient of increasing buffer C (buffer B, 1 M NaCl). Peak fractions were pooled and after concentration by Amicon Ultra tubes (Millipore, MWCO 10 kDa) subjected to gel-filtration (Superose 6, Amersham) in buffer B. Protein preparation were normalized by Flag ELISA as follows, 96 well plates (PolySorp, Nunc) were coated for 1 h at 37 °C with 200 μl of 10-fold dilutions of the crude lysates after the expression of the Flag-peptides. The coating solution was drained and wells were blocked with 200 μl TBS–T (Tris-buffered saline, 0.05% Tween 20) containing 5% nonfat dry milk for 30 min at RT. Plates were then washed once with 200 μl TBS–T and incubated for 1 h at RT with a 100 μl aliquot of Anti-FLAG M2-Peroxidase antibody (Sigma), diluted 1:10000 in TBS–T. After three washing steps with TBS–T, the ELISA was developed using 3,3′,5,5′ tetramethylbenzidine (TMB; Sigma). Hundred microliter TMB-solution (20 μg/ml TMB and 0.01% H_2O_2 in 40 mM citric acid, pH 3.95) were added to the wells and the reaction was stopped after a few minutes by 50 μl of 1 M H_2SO_4. Absorbance was read at 450 nm.

2.5. In vitro β-lactamase protein fragment complementation assay (iPCA)

In a total volume of 200 or 225 μl, 20 μl of the NUL50-His eluates (100 μg total protein) and CUL53-His eluates (25 μg total protein) were combined in a 96 well plate and mixed with 120 μl Na–phosphate buffer (0.1 M, pH 7.0), 15 μl 500 mg/ml nitrocefin (Oxoid) and 25 μl H_2O. The above described ratio of NUL50-His and CUL53-His eluates was established by testing for the highest activity using a setup, in which both components were applied in different amounts (25, 50, or 100 μg total protein, data not shown). For inhibition experiments H_2O was replaced by 25 μl of synthesized peptides (0.1% TFA in H_2O) or the 50 μl anti-Flag M2 agarose with purified Flag-polypeptides. Thereby, the NUL50-His, the peptide solutions, and the CUL53-His eluate were pre-mixed and nitrocefin was added to start the assay. In a Versamax plate reader (Molecular Devices) the change in absorption at 495 nm as a measure of nitrocefin hydrolysis was observed over 20 min at 37 °C. The maximal change in absorption (vmax) was determined by the data points in the linear range and expressed in milliabsorption units/minute. All experiments were repeated three times in technical triplicates; except the peptide inhibition assay shown in Fig. 4C, in which no technical replicates were made.

2.6. Protein analysis

Protein concentration was measured by the BCA or Bradford method. For analysis, 15 μl of the samples were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Proteins were either stained by Coomassie Blue or transferred from the gel onto Hybond-P membranes (Amersham Biosciences) in the presence of blotting buffer (25 mM Tris, 192 mM glycine, 20% [vol/vol] methanol, pH 8.3). Membranes were blocked in TBS–T containing 5% nonfat dry milk over night at 4 °C. To detect the HA- and Flag-tagged constructs, the membrane was then incubated at room temperature for 1 h with TBS–T containing Anti-HA-Peroxidase High Affinity antibody (Roche, 1:5000) or Anti-FLAG M2-Peroxidase antibody (Sigma, 1:2000). Membranes were washed with TBS–T and proteins were visualized with an ECL-Plus Western Blot detection system (GE Healthcare).

3. Results

3.1. Co-purification of pUL50 and pUL53 subunits

pUL50 and pUL53 interact during HCMV morphogenesis to mediate the nuclear egress of virus capsids (Milbradt et al., 2007; Muranyi et al., 2002; Schnee et al., 2006). We and others determined the domains in both proteins which are essential for this interaction in cells (Sam et al., 2009; Schnee et al., 2006). To confirm that this interaction takes place between the identified protein fragments without the contribution of other viral or host cell proteins and to reduce the size of the interacting proteins for the development of an *in vitro* PCA, truncated versions of pUL50 and pUL53 were co-expressed in *E.coli* (see Supplementary Fig. S1) .

As interacting fragment of pUL50, the N-terminal 172 amino acids (aa) were chosen (UL50-His; aa1–172), harboring the predicted binding site to pUL53 previously shown to be located in the first 169aa of the protein (Sam et al., 2009; Schnee et al., 2006) but lacking the predicted transmembrane part of the variable region (Bubeck et al., 2004). UL50(aa1–172) was C-terminally His-tagged and cloned downstream to non-tagged fragments of pUL53, covering the aa57–92 (UL53_1) or 57–129 (UL53_2), representing a part of the first conserved region (CR1) of pUL53 with the predicted binding site to pUL50 (Sam et al., 2009; Schnee et al., 2006) or the whole CR1 (59–127; Schnee et al., 2006), respectively (for schematic representation of the fragments see Fig 1A). The expression of both ORFs was driven by a separate ribosomal binding site (RBS) (Supplementary Fig. S1A). After expression of the bicistronic constructs, the pUL50/pUL53 complex was subsequently purified with Ni^{2+}–NTA, anion exchange chromatography and gel filtration. For all purification steps Coomassie-stained gels show the protein bands at the predicted heights of 20.6, 4.5 and 8.7 kDa for UL50-His, UL53_1 and UL53_2, respectively (Supplementary Fig. S1B). After the first purification step both short pUL53 fragments co-purified with UL50-His and little impurities of other bacterial proteins were detectable. During anion exchange chromatography UL50-His and UL53_1 eluted at low salt conditions with 8% of buffer B that contained 1 M NaCl (Supplementary Fig. S1C). From gel filtration, the UL50-His/UL53_1 complex eluted also with a clear peak in three successive fractions (Supplementary Fig. S1D). Despite a significant

loss of the UL53_1 over the three subsequent purification steps, the data showed that the expressed fragments of pUL50 and pUL53 on their own are sufficient to form a complex.

3.2. Establishment and validation of the in vitro PCA (iPCA)

To test whether the two protein domains are able to interact *in vitro*, we wanted to set up a PCA using purified proteins. In a PCA two non-functional fragments of a reporter protein are fused to the two proteins of interest. If the fragments reach proximity due to the interaction of the proteins to which they are fused, folding of the native conformation leads to reconstitution of reporter activity (Fig. 1B). To set up a PCA *in vitro* (iPCA) the *E.coli* TEM-1 β-lactamase (Bla) was split in two parts according to the cell based PCAs (Galarneau et al., 2002), representing the N-terminus from aa24 to 194 (N, BlaN) and the C-terminus from aa196 to 286 (C, BlaC). The Bla fragments were HA-tagged and N-terminally fused to the truncated HCMV pUL50 and pUL53 proteins that were C-terminally His-tagged, giving rise to NUL50-His, and CUL53-His (Fig. 1C). As truncated version of pUL50 the aa residues 1–172 corresponding to the complex purification were used, whereas for pUL53 the residues 1–129, which include the previously expressed CR1 and additionally the variable region in the N-terminus (see Fig 1A), were processed. As a negative control for the binding assay, the N-terminal Bla fragment was also fused to a designed non-binding mutant of pUL50, covering as well the first 172 residues, but lacking aa51–57 as the predicted binding motif to pUL53 (NUL50DM-His) (Schnee et al., 2006).

After separate expression and Ni^{2+}–NTA purification of the three constructs, the eluted proteins were visualized by Coomassie-staining (Fig. 1D) and an HA-specific Western Blot (Fig. 1E). The constructs were detected at the predicted molecular weights of 42 kDa (NUL50-His and NUL50DM-His) and 28 kDa (CUL53-His) with certain background impurities. Furthermore, the Western Blot showed equal amounts of NUL50-His and NUL50DM-His in the protein samples.

Combining eluates of CUL53-His with NUL50-His resulted in a strong Bla activity, monitored by hydrolysation of the Bla substrate nitrocefin, whereas CUL53-His combined with NUL50DM-His or each of the protein extracts alone did not lead to a significant Bla activity (Fig. 2A). After the combination of NUL50-His and CUL53-His lactamase activity was at least 10 times higher than for any of the negative controls. Next, the specificity of the NUL50-His/CUL53-His iPCA was tested in the presence of increasing amounts of competing UL50-His. UL50-His, expressed individually from a pET24 derived vector and purified by the His-tag, binds CUL53-His but cannot complement Bla function due to the lack of the Bla-fusion fragment. Accordingly, the PCA signal decreased in a UL50-His concentration dependent manner. The initial hydrolysis rate of 70 absorption units/min in the absence of UL50-His was gradually reduced to more than half, whereas BSA in the same concentration did not lead to a decrease of the Bla signal (Fig. 2B).

The background of the NUL50-His/CUL53-His iPCA, due to spontaneous folding of the Bla-fragments or contaminant of bacterial β-lactamase like activities was relatively low. Furthermore, the iPCA was dose dependently inhibited by untagged pUL50 fragment. Thus we considered the *in vitro* PCA (iPCA) to be useful to characterize the interaction of pUL50 and pUL53 and to test for inhibitors.

3.3. Identification of a linear UL50 binding motif in UL53

pUL50 and pUL53 belong to the highly conserved pUL34 and pUL31 protein family, respectively. The binding of pUL31 homologs to pUL34 homologs was shown to be herpesvirus subfamily specific, however, in all studied pUL31 proteins, the binding site was located in the first conserved region (Schnee et al., 2006). The

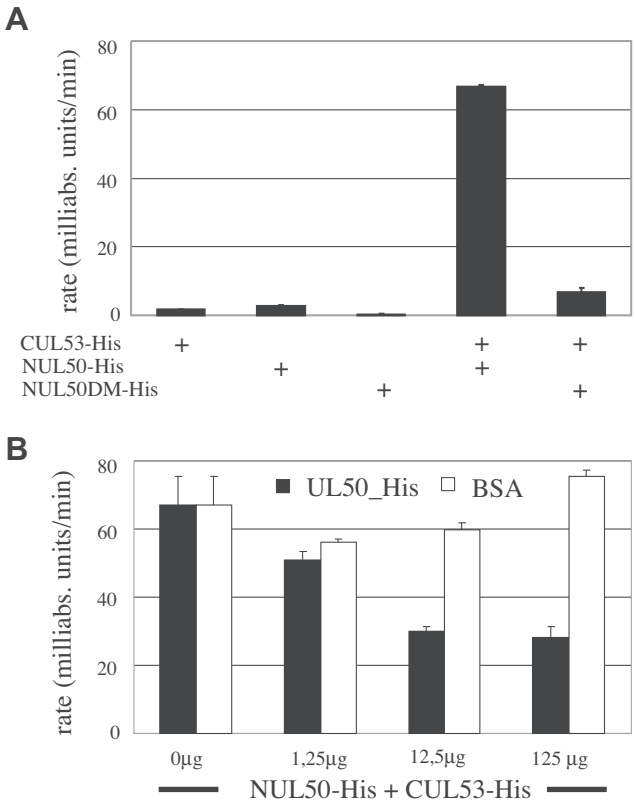

Fig. 2. Characterization of NEC–iPCA (A) 100 µg of the NUL50-His and 25 µg of the CUL53-His protein preparation were mixed and the lactamase substrate, nitrocefin, was added. Mean nitrocefin hydrolysis rates were determined and shown as rate in milliabsorption units/min. Below the plot the protein preparation present in the tests are indicated. (B) 100 µg of the NUL50-His and 25 µg of the CUL53-His protein preparation were mixed with increasing amounts of inhibitory UL50_His (lacking the BlaN-tag) or BSA and nitrocefin was added. Mean nitrocefin hydrolysis rates were determined and shown as rate in milliabsorption units/min.

alignment of the CR1 of 36 pUL31 protein family members of all three subfamilies shows the conservation of three cysteines and one proline throughout the families (Supplementary Fig. S2). The N-terminal part of the CR1 seems less conserved, however, an alignment of proteins of each subfamily separately highlights more conserved residues in this part, as shown for the β-herpesvirus subfamily in Supplementary Fig. S2. This further emphasizes the subfamily specificity of binding. The binding site in pM53, the homolog in MCMV, was located to the first, in general less conserved part of CR1 (Lotzerich et al., 2006) and the site was confirmed by an *in vitro* study on pUL53 (Sam et al., 2009), which shows a high degree of conservation in the alignment exclusively run on pUL31 proteins of the β-herpesvirus subfamily.

To narrow down the binding site in pUL53 and proof of principle of the iPCA as inhibitor screening system, short polypeptides mimicking sequences of pUL53 were expressed in bacteria. These polypeptides with an N-terminal Flag-tag were designed to test their ability to compete for NUL50-His in the iPCA. Prior to purification, the bacterial lysates were normalized for their total protein content and their proportion of Flag-tagged protein, which was determined by an anti-Flag-ELISA. After purification by the anti-Flag-matrix, matrix bound Flag-tagged polypeptides were added to the NUL50-His/CUL53-His iPCA to inhibit the interaction, visualized by the decrease of the signal produced by the active Bla enzyme. Flag-tagged polypeptides that mimicked the CR1 of pUL53 were able to inhibit the NUL50-His/CUL53-His interaction (Fig. 3A). In the presence of matrix bound Flag-tagged polypeptides, the signal in the PCA was reduced to 50% of the signal ob-

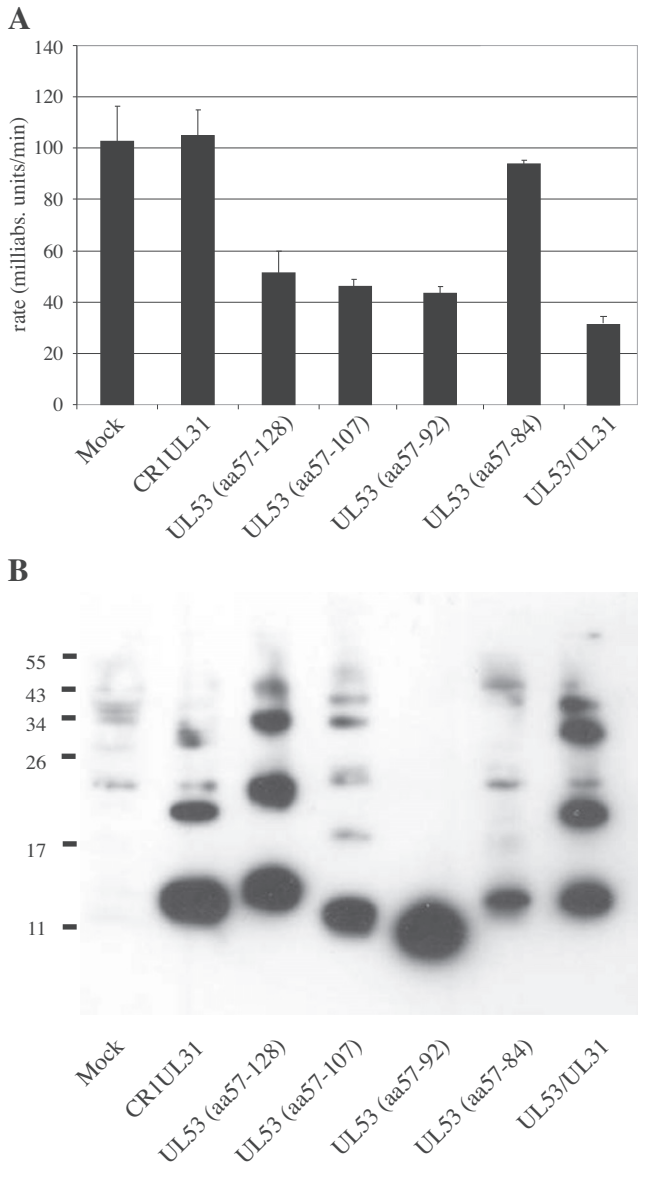

Fig. 3. The pUL50/pUL53 interaction can be inhibited by Flag-tagged sub-domains of pUL53 (A) Bla activity after incubation of NUL50-His and CUL53-His with matrix bound Flag-peptides. Mean nitrocefin hydrolysis rates were determined and shown as rate in milliabsorption units/min. (B) Expression of the Flag-peptides detected by Flag-specific Western Blot after normalization and separation of the indicated samples on a 15% SDS–PAGE. Numbers indicate the position and size of the protein marker in kDa.

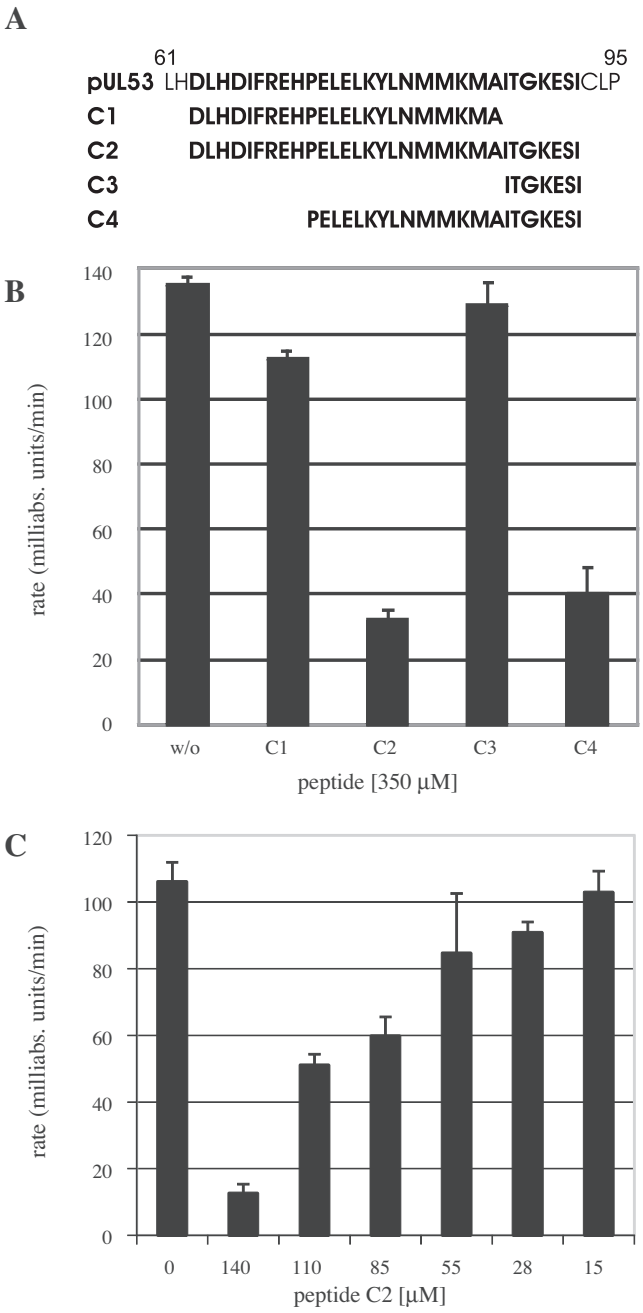

Fig. 4. The pUL50/pUL53 interaction can be inhibited by a 21aa peptide (A) Schematic representation of the peptides used in this study (C1–4) aligned to the wild type pUL53 sequence from aa61 to 95 (upper row). (B) Bla activity after incubation of NUL50-His and CUL53-His with synthetic peptides. Mean nitrocefin hydrolysis rates were determined and shown as rate in milliabsorption units/min. (C) Bla activity after incubation of NUL50-His and CUL53-His with decreasing concentrations of synthetic peptide C2. Nitrocefin hydrolysis rates of duplicates were determined and mean values are shown as rate in milliabsorption units/min.

tained when protein lysate lacking Flag-tagged polypeptides (mock) was incubated with the matrix as negative control. The homologous CR1 of pUL31 of HSV1 (UL31 (aa55–128)) or the very short fragment of pUL53, UL53(aa57–84), however, did not influence the interaction of the HCMV proteins. It is possible that using this approach we could not reach the necessary concentration for UL53(aa57–84) which would be needed for measurable inhibition (Fig 3B).

Since it has been shown by earlier studies on the pM53 homolog (Lotzerich et al., 2006) and mutagenesis studies on purified pUL53 proteins (Sam et al., 2009) that the amino acid stretch corresponding to aa77–84 of pUL53 carries residues involved in binding to pUL50/pM50, amino acids downstream to aa84 were replaced by the homologous CR1 residues of pUL31 of HSV1, which itself does not bind pUL50. The resulting chimera (UL53/UL31) inhibited the NUL50-His/CUL53-His interaction by 70% (Fig. 3A). All Flag-tagged constructs were detected at the predicted size except the pUL53(aa57–84), which migrated slower than its predicted size (4.9 kDa). (Fig. 3B).

3.4. Synthetic peptides derived from the CR1 of UL53 inhibit the NEC–iPCA

Determination of Flag-tagged polypeptides in the bacterial lysates showed considerable differences in expression of the peptides or their stability. The lysates obtained from CR1UL53

expression (UL53 (aa57–128)), as the sample with the highest proportion of Flag-tagged peptide, contained about 10 times more Flag-tagged peptide than lysates obtained from the expression of CR1UL31 (UL31 (aa55–128)) or UL53(aa57–84) (data not shown). The dilution of the sample, necessary to normalize the samples, impurities from bacterial expression of the Flag-tagged peptides and a limit in length of peptide to be expressed in bacteria show the restrictions of the system. To circumvent these limitations, synthetic peptides were used for further experiments.

First two peptides were tested that match pUL53 from aa63 to 85 (C1) and aa63 to 92 (C2) (Fig. 4A). As seen for the Flag-tagged peptides, only the long C2 peptide, corresponding with its C-terminus to UL53(aa57–92) inhibited the NUL50-His/CUL53-His interaction significantly, whereas C1 did not. When C2 was present in the iPCA at a concentration of 350 μM, the PCA signal was reduced to 24% of the signal obtained in the absence of peptide (Fig. 4B). When C2 was added to the assay in lower concentrations, the inhibitory effect decreased in a concentration dependent manner, showing the specificity of the inhibition (Fig. 4C). A short peptide, covering only the seven additional amino acids present in the C2 peptide (aa86–92; C3), was next tested for an inhibitory effect on the interaction but did not influence the assay (Fig. 4B). Having determined the prolonged part of C2 as necessary but not sufficient for disruption of the NUL50-His/CUL53-His interaction, a fourth peptide was designed, covering pUL53 from aa72 to 92 (C4), which again was able to interfere with the interaction and reduced the PCA signal to 30% (Fig. 4B). The disruption of the NUL50-His/CUL53-His interaction by a 21aa peptide shows the importance of this stretch of residues in the pUL53 protein and indicates a continuous binding motif.

4. Discussion

β-lactamase based PCA is predominantly used *in vivo* (Michnick et al., 2007). Proteins can be studied in the natural context, ensuring subcellular targeting, post-translational modifications and interactions with other proteins required for correct functioning. Taking these advantages into account, PCAs were proposed as suitable candidates for cell based high-throughput screens for interacting proteins or inhibitors. (Remy and Michnick, 2004) reported a GFP–PCA based functional cDNA library screen for interaction partners of the protein kinase PKB/Akt. This shows that PCA allows combination of a genome wide cell based screen and functional validation experiments within one system. The work presented here is the first set up for a β-lactamase based cell free PCA (*in vitro* PCA; iPCA) working with a defined protein–protein interaction using purified components. To the best of our knowledge, this shows for the first time that the same assay system could be used for characterization of a protein–protein interaction in its biological context (Schnee et al., 2006) and in a completely cell free setting (this study). Thus, the Bla–PCA allows *in vivo* analyses as well as targeted, biochemically defined and cost effective investigations *in vitro* combining the advantages of both areas of application. This provides the opportunity to translate the results of systemic cell based high-throughput screens to a simple but quantitative *in vitro* assay.

Here, as an example, we analyzed the interaction between the pUL50 and pUL53 of HCMV by iPCA. These proteins are members of the pUL34 and pUL31 families of conserved herpesvirus proteins, respectively. These proteins form a complex at the inner nuclear membrane, coined nuclear egress complex (NEC), which is crucial for virus growth (Johnson and Baines, 2011). The here presented iPCA for the HCMV homologs allowed us to analyze this interaction between the cytomegalovirus NEC subunits in a cell free system. We show that the two NEC proteins of HCMV are sufficient to form a complex *in vitro* in agreement with a recent study which used native purified proteins (Sam et al., 2009). In addition, using iPCA we showed that the main binding motif of this interaction is found in a linear motif of pUL53. Interestingly, we could confirm the predictions (Sam et al., 2009; Schnee et al., 2006) that the specific binding site of pUL53 to pUL50 is located between aa72 and 85. However, the peptide representing this motif alone was not sufficient to inhibit the NEC–iPCA. The presence of a C-terminal sequence was required comprising the aa86–92. While the first motif was not conserved between HCMV and HSV-1 homologs the second motif from HSV-1 could replace the second HCMV motif provided as a chimeric protein fragment (see Fig. 3A second and last column, respectively). This data indicate that the binding site of the pUL53 homologs to the pUL50 homologs are bipartite comprising an N-terminal part which is probably directly involved in the interaction confirming that the specific binding site is located within the predicted alpha-helix between the aa61 and 82 (Sam et al., 2009) However, our data also indicate that a second part is also required (aa83–92) which may play a rather indirect, structural role.

These data together with previous studies indicating independent folding of the NEC proteins in cells suggests that the pUL50–pUL53 interaction is non-obligatory from the point of view of protein folding (De et al., 2005) and is mediated by surface with a limited volume. Therefore we can postulate the pUL50–pUL53 interaction as a new target for screening new antivirals. In fact, the here described iPCA assay is compatible with high-throughput screens for small molecular inhibitors without major further modifications.

The Bla-tagged minimal interaction fragments of pUL50 and pUL53 of HCMV were expressed in *E. coli* and purified by the introduced C-terminal His-tags. The Bla-tagged proteins successfully interacted in the PCA when the protein eluates were mixed. Thereby, the background of irrelevant proteins in the extracts did not hamper the detection of the interaction. The Bla activity measured for the interaction was more than 10 times higher than the background, which was defined by the Bla activity in the protein samples tested separately and in combination with a BlaN-tagged pUL50 mutant deficient in pUL53 binding (Bubeck et al., 2004). Future high-throughput studies will demand for scale down of protein amounts. In this work, we used a relatively high amount of proteins resulting in a strong Bla activity. However, a specific, although reduced Bla activity might also be anticipated when lower protein amounts will be applied.

In our previous study we showed that the cell based Bla–PCA is applicable for the analysis of the NEC of all tested herpesviruses. It is reasonable to speculate that the iPCA will as well be a useful set up to characterize the NECs of other herpesviruses. We believe that NEC of herpes simplex virus 1 and pseudorabies virus is characterized well enough (Klupp et al., 2007; Roller et al., 2010; Schnee et al., 2006) for plausible prediction of a similar binding fragment and therefore should be amendable for similar iPCA tests. Moreover, we believe that viral interactomes can be generated using cell based Bla–PCA screens to identify a set of interactions, which should then be confirmed in the viral context and transferred to iPCA. In most of the cases the conversion between the cell based PCA and iPCA will need mapping work to define the interaction domains, which can be fused to Bla-fragments and expressed in *E. coli*. Yet, the presented data on NEC–iPCA might serve as proof of principle to generate a new platform for drug discovery.

5. Conclusion

The presented cell free PCA showed that the core interaction of the human cytomegalovirus nuclear egress complex relies on a lin-

ear epitope. This new pUL50–pUL53 interaction assay allows screens for inhibitors. We describe synthetic peptides derived from the CR1 of pUL53 which can specifically inhibit the NEC–iPCA.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.antiviral.2012.04.009.

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