Short Communication

Multimerization of human cytomegalovirus regulatory protein UL69 via a domain that is conserved within its herpesvirus homologues

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The UL69 protein of human cytomegalovirus is a multifunctional regulatory protein that has counterparts in all herpesviruses. Some of these proteins have been shown to function primarily at the post-transcriptional level in promoting nuclear export of viral transcripts. Consistently, this group has reported recently that pUL69 is an RNA-binding, nucleocytoplasmic shuttling protein that facilitates the cytoplasmic accumulation of unspliced mRNA via its interaction with the cellular mRNA export factor UAP56. Evidence has been presented to suggest that some of the pUL69 homologues self-interact and function in vivo as multimers. Herein, the possibility of pUL69 self-association was examined and it has been demonstrated that pUL69 can interact with itself in vitro and in vivo in order to form high-molecular-mass complexes. The self-interaction domain within pUL69 was mapped to a central domain of this viral protein that is conserved within the homologous proteins of other herpesviruses, suggesting that multimerization is a conserved feature of this protein family.

Nevertheless, several functions can be linked to this region that seem to be specific for either of the herpesviral mRNA export factors. For instance, the C-terminal half of ICP27 contains three putative KH-like domains that are postulated to contribute to the RNA-binding specificity of ICP27 (Soliman & Silverstein, 2000); furthermore, the C terminus is required for binding to splicing factors and to the cellular mRNA export receptor TAP (Sciabica et al., 2003; Chen et al., 2005). The homology region within pUL69 has been identified as a binding domain for the transcription elongation factor hSPT6 (Winkler et al., 2000); in ORF57 of KSHV the respective region has been shown to be required for binding to CK2 kinase (Malik & Clements, 2004). Thus, as a common theme, the conserved homology region appears to be involved in protein–protein interactions.

Evidence has been presented to suggest that at least some of the pUL69 homologues self-interact via their conserved homology region and function in vivo as multimers (Zhi et al., 1999; Wadd et al., 1999; Malik & Clements, 2004). Due to these findings we examined the possibility of pUL69 self-association. To be able to uncover a putative interaction by co-immunoprecipitation experiments, we first constructed myc-tagged eukaryotic expression vectors encoding either pUL69 (m-UL69) or pUL84 (m-UL84) (Lischka et al., 2003). These constructs were cotransfected into HEK293T cells together with the reciprocal plasmids expressing pUL69 and pUL84 as in-frame fusions with the

The human cytomegalovirus protein encoded by the ORF UL69 belongs to a family of regulatory factors that is conserved among all herpesviruses and includes the proteins ICP27 of herpes simplex virus type I, EB2 of Epstein–Barr virus and ORF57 of Kaposi's sarcoma-associated herpesvirus. For all characterized members of this protein family, a function as post-transcriptional activators that facilitate the nuclear export of mRNA has been documented. This is mediated via the capability of these proteins to shuttle between the nucleus and the cytoplasm and to interact with components of the cellular mRNA export machinery (for reviews see Cullen, 2003; Sandri-Goldin, 2004; Boyne & Whitehouse, 2006; Lischka & Stamminger, 2006). Furthermore, all these proteins are able to interact directly with RNA (Mears & Rice, 1996; Sandri-Goldin, 1998; Hiriart et al., 2003; Toth et al., 2006). The UL69 protein and its counterparts share a conserved homology region that is localized at the C terminus of the α- and γ-herpesvirus factors, whereas, due to a unique C-terminal protein domain, this region can be found in the central part of pUL69 (Fig. 1a). Interestingly, most of the protein domains with importance for mRNA export stimulation (e.g. interaction site for cellular mRNA export factors, nuclear export signal) have been mapped to regions of the herpesviral mRNA export factors that show either no or low sequence conservation (Fig. 1a). In contrast, no common functional domains shared by all protein family members have yet been assigned to the homology region.
Fig. 1. (a) Diagrams of the predicted secondary structures of pUL69 and its herpesviral homologues. The PSIPRED program was used to create the diagrams (Jones, 1999). White boxes, predicted α-helices; black boxes, predicted β-sheets; grey rectangle, ICP27 homology region; NES, nuclear export signal; RBD, RNA-binding domain; REF, TAP, UAP and hSPT6, binding sites of the respective cellular factors; KH1–3, putative KH RNA-binding motifs; SID, self-interaction domain of ICP27. (b) Self-interaction of pUL69. HEK293T cells were cotransfected with a combination of two plasmids encoding either myc- and FLAG-tagged pUL69 (F-UL69, m-UL69, lanes 1, 3, 4, 5, 7 and 8), myc-tagged pUL69 and FLAG-tagged pUL84 (lane 2) or FLAG-tagged pUL69 and myc-tagged pUL84 (lane 6). Protein in the input was analysed by Western blotting (upper panel). Immunoprecipitation was performed using different monoclonal antibodies: lanes 1–3, anti-FLAG; lanes 5–7, anti-myc; lanes 4 and 8, anti-HA. Co-immunoprecipitated proteins were visualized by Western blotting using either anti-myc (lanes 1–4) or anti-FLAG (lanes 5–8) antibodies. RNase, RNase treatment of cell extracts. (c) Immunopurification of FLAG-tagged pUL69 from mammalian cells. F-UL69 was purified from transfected HEK293T cells by anti-FLAG M2 agarose affinity chromatography. Increasing amounts of the purified protein were subjected to SDS-PAGE followed by Coomassie-blue staining and Western blotting. (d) Chemical cross-linking of pUL69. Purified pUL69 was kept untreated (0 min) or treated for 2, 5, 10 and 20 min with GA followed by SDS-PAGE and Western blotting using a pUL69 serum. (e) Western blots showing the gel filtration elution profile of purified pUL69. The void volume and elution position of the Amersham HMW/LMW size markers are indicated, as is a molecular mass marker for the Western blot. RNase, protein fractions were treated with 100 μg RNase A ml⁻¹ prior to gel filtration.
FLAG epitope (F-UL69 and F-UL84; Fig. 1b, input) (Lischka et al., 2003, 2006). Subsequently, co-immunoprecipitation experiments were performed using either a monoclonal antibody directed against the FLAG (M2) or the myc (9E10) epitope (IP-FLAG, IP-myc). Co-immunoprecipitated proteins were analysed by Western blotting. As shown in Fig. 1(b), lower panel, lanes 1 and 5, m-UL69 was efficiently co-immunoprecipitated with F-UL69 and vice versa. This interaction was specific because (i) pUL69 did not co-precipitate with the control protein pUL84 (lanes 2 and 6).

Fig. 2. Mapping of the self-association domain within pUL69 by yeast two-hybrid analysis. (a) Qualitative analysis of pUL69 self-interaction after staining for β-galactosidase activity. Yeast cells were transformed with two expression plasmids, one of which encoded either BD-UL69 or the GAL4 DNA-binding domain alone (BD). The second plasmid encoded either pUL69 as a fusion with the GAL4 activation domain (AD-UL69) or the GAL4 activation domain alone (AD). Murine p53 (BD-p53) and SV40 TAg (AD-TAg) served as a positive control. (b) N- and C-terminal deletion mutants as well as internal deletion mutant Δ478-527 and mutant C495A of pUL69, generated as in-frame fusions to the GAL4 DNA-binding domain, are indicated. The location of the NLS and NES, the binding sites for UAP56 (UAP56-BD) and hSPT6 (hSPT6-BD), and the RNA-binding domain (RNA-BD) containing arginine-rich sequences (R1, R2, RS) is depicted (Winkler et al., 2000; Lischka et al., 2001, 2006; Toth et al., 2006). (c) Yeast cells were transformed with a combination of vectors encoding the pUL69 mutants (shown in b) and wild-type pUL69 fused to the GAL4 activation domain followed by analysis of β-galactosidase expression by filter lift assays.
and (ii) no signal was present when a non-specific control antibody was used for precipitation (lanes 4 and 8). Since pUL69 is an RNA-binding protein, we also carried out identical experiments after digestion with RNase A. As shown in Fig. 1(b), lanes 3 and 7, treatment with 100 μg RNAse A ml⁻¹ did not affect pUL69 co-precipitation, indicating that pUL69 self-association did not occur through RNA-bridging. To address the question whether pUL69 forms dimers or multimers, the effect of glutaraldehyde (GA) as an inducer of covalent cross-links between interacting proteins was assessed. For this, we first purified pUL69 as a FLAG-fusion protein from transfected HEK293T cells using an anti-FLAG

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**Fig. 3.** Nuclear recruitment of a pUL69 NLS-mutant after co-expression of wild-type pUL69. (a) Schematic representation of GFP-tagged wild-type pUL69 and the FLAG-tagged pUL69 alanine replacement mutant UL69mNLS2. A part of the bipartite pUL69 NLS is shown in the expanded section at the bottom; alanine replacement mutations introduced into the pUL69 NLS are indicated. (b) Co-immunoprecipitation analysis of GFP-UL69 with either wild-type UL69 or alanine replacement mutant UL69mNLS-2. HEK293T cells were cotransfected with a plasmid for GFP-UL69 together with plasmids for either a FLAG-tagged IE2 deletion mutant (lane 1, F-ΔIE2), FLAG-tagged wild-type pUL69 (lane 2, F-UL69) or FLAG-tagged alanine replacement mutant UL69mNLS-2 (lanes 3 and 4, F-UL69mNLS-2). The amount of protein in the input was determined by Western blotting (upper panel, α-GFP; middle panel, α-FLAG). Immunoprecipitation was performed either with monoclonal antibody anti-FLAG M2 (IP-FLAG) or with an anti-actin antibody (IP-C). Co-precipitated GFP-UL69 was detected by Western blotting (lower panel). (c) HeLa cells were transfected with plasmids encoding GFP-UL69 and UL69mNLS2, either singly or in the indicated combination, followed by indirect immunofluorescence analyses. Wild-type pUL69 was visible through its GFP-moiety (GFP), UL69mNLS2 was detected with an anti-FLAG monoclonal antibody (αFLAG). DAPI, DAPI-staining of the respective cell nuclei.
affinity gel (Toth et al., 2006). As shown by Coomassie blue staining and Western blotting, three characteristic pUL69 isoforms were purified that correspond to differentially phosphorylated protein isoforms (Fig. 1c) (Winkler & Stamminger, 1996). GA efficiently forms covalent bonds between lysine residues and thus stabilizes multimeric forms of proteins that can be subsequently visualized by SDS-PAGE (Mische et al., 2005). As shown in Fig. 1(d), no higher-order form of pUL69 was evident in the absence of cross-linker (0 min), whereas exposure of purified protein to 0.1% GA resulted in the progressive formation of SDS-resistant multimers in a time-dependent manner (2–20 min). Interestingly, apart from very high molecular mass entities, no band consistent with a pUL69 dimer (~220 kDa) could be detected. This result supports the idea that pUL69 is engaged more in the formation of multimers than dimers. To prove this, we subjected pUL69 to gel filtration analyses (Fig. 1e). For this, pUL69, purified from transfected mammalian cells, was fractionated on a Pharmacia Superdex 200 column in 150 mM NaCl, 50 mM Tris/HCl (pH 7.5), 10% glycerol. Fractions (0.5 ml) were collected and analysed by SDS-PAGE and Western blotting using a polyclonal pUL69-specific rabbit serum. Protein molecular mass standards (HMW/LMW gel filtration calibration kits; Amersham Biosciences) were used to calibrate the column. Examination of pUL69 in the fractions showed that the protein elutes in a heterogeneous high molecular mass population ranging from 438 kDa up to >2 MDa. Importantly, consistent with the cross-linking experiments, no pUL69 monomers (~110 kDa) or dimers (~220 kDa) could be detected. RNase treatment of the purified protein prior to gel filtration did not induce a significant change of the elution profile (Fig. 1e, lower panel). Taken together, these experiments strongly suggest that (i) pUL69 has the capacity to self-interact, (ii) pUL69 self-interaction is not bridged by RNA and (iii) pUL69 forms multimeric complexes in mammalian cells.

To further evaluate the self-association of pUL69 in vivo, we next performed interaction studies using the yeast two-hybrid system exactly as described by Winkler et al. (2000). Saccharomyces cerevisiae strain Y153 was co-transformed with expression plasmids encoding pUL69 fused either to the GAL4 activation domain (AD-UL69) or to the GAL4 DNA-binding domain (BD-UL69). Subsequently, the activation of the β-galactosidase reporter gene was monitored by filter lift experiments. As shown in Fig. 2(a), an in vivo interaction of pUL69 with itself could be observed (Fig. 2a, lane 5). This interaction was considered specific since control transformations of AD-UL69 or BD-UL69 with the appropriate empty GAL4-plasmids excluded self-activation of the reporter gene by pUL69 (Fig. 2a, lanes 2 and 3). Next, we sought to identify the interaction motif within pUL69. To uncover this domain, a series of N- or C-terminal pUL69 deletion mutants fused to the GAL4 DNA-binding domain was constructed. These mutants were analysed in the yeast two-hybrid assay for their interaction with AD-UL69. Fig. 2(b) and (c) summarize the results of these experiments. They show that a central domain within pUL69, spanning residues 269–574, is both required and sufficient for efficient self-interaction. Interestingly, this region exactly corresponds to a domain within pUL69 that mediates binding to the cellular transcription elongation factor hSPT6 (Winkler et al., 2000). In addition, an internal deletion of a cysteine-rich region between amino acids 478 and 527, a region that is conserved within the homologous proteins of other herpesviruses, or a mutation of cysteine-495, which has previously been shown to abrogate the interaction with hSPT6, also abrogated pUL69 self-interaction (Winkler et al., 2000). These findings suggest that the integrity of the identified pUL69 domain is either essential for proper folding and protein stability and/or that pUL69 self-interaction is a prerequisite for hSPT6 binding.

Given our findings that pUL69 self-associates in both biochemical assays and in yeast cells, we finally asked whether pUL69 also interacts with itself in the context of a mammalian cell. To address this question we first generated two eukaryotic expression plasmids, GFP-UL69 and UL69mNLS2. GFP-UL69 encodes wild-type pUL69 fused to GFP; UL69mNLS2 encodes a pUL69 FLAG-fusion protein that carries four alanine substitutions within its bipartite nuclear localization sequence (NLS) (Fig. 3a). The respective proteins were still able to self-interact as analysed by co-immunoprecipitation experiments (see Fig. 3b). Next, we determined the subcellular localization of each protein in transfected HeLa cells by indirect immunofluorescence analyses. As depicted in Fig. 3(c), panels 1–3, GFP-UL69 was detected exclusively in the nucleus, whereas UL69mNLS2, due to the replacement of four NLS-determining basic residues by alanine, displayed a partial cytoplasmic localization (Fig. 3c, panels d–f). However, as described above (Fig. 2), the N-terminal part of pUL69, including the NLS, is not required for pUL69 self-interaction. Taking this into consideration, we reasoned that co-expression of UL69mNLS2 and the strictly nuclear-localized GFP-UL69 would increase the nuclear localization of the mutant protein by heterodimer formation. To verify this, HeLa cells were cotransfected with GFP-UL69 and UL69mNLS2. Subsequently, the localization of co-expressed proteins was assessed by indirect immunofluorescence. Due to the presence of two different tags (GFP and FLAG, see Fig. 3a), the wild-type protein could be easily distinguished from the mutant protein within the same cell. For GFP-UL69, nuclear staining was detected, as expected (Fig. 3c, panel h). However, when the subcellular localization of UL69mNLS-2 was assessed, the majority of cells were found to have a predominant nuclear fluorescence with no or only a slight cytoplasmic staining (Fig. 3c, panel i). Since this staining pattern was never observed with UL69mNLS-2 alone, these data strongly suggest heterodimer/multimer formation between GFP-UL69 and the pUL69 mutant in the context of mammalian cells.

Taken together, our data have shown that pUL69 self-associates and forms multimers both in vitro and in vivo. We
have demonstrated that pUL69 self-association is apparently not dependent on functional motifs involved either in intracellular transport (NLS, NES) or UAP56 and RNA binding (UAP56-BD, RNA-BD) (Fig. 2b, c), since the respective regions could be deleted without affecting pUL69 self-interaction. This is also supported by our previous notion that C-terminal pUL69 deletion fragments lacking the self-interaction domain still demonstrate RNA binding and UAP56/URH49 interaction (Lischka et al., 2006; Toth et al., 2006). Thus, we present strong evidence to suggest that self-association is a shared property of all members of the ICP27 protein family that appears to be mediated by the conserved homology region. A comparison of the predicted secondary structure of the respective proteins using the PSIPRED method (Jones, 1999) supports the assumption that the homology region may form a globular, highly structured core domain (Fig. 1a). One may speculate that this structure forms a central scaffold for both self-interaction and binding to other specific proteins. Due to structural constraints, this domain may have been less amenable to divergence during co-evolution of the different herpesviruses, whereas other regions of these proteins were able to adapt to the specific requirements that these proteins have to fulfil during the life cycle of the individual viruses. Further studies aimed at the determination of the three-dimensional structure of these proteins will be required to ascertain this hypothesis.

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References


