Virology 422 (2012) 308–316

Contents lists available at SciVerse ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

Identification of a single amino acid residue which is critical for the interaction between HSV-1 inner tegument proteins pUL36 and pUL37

Barbara J. Kelly [a], Branka Mijatov [a], Cornel Fraefel [b], Anthony L. Cunningham [a], Russell J. Diefenbach [a,*]

[a] Centre For Virus Research, The Westmead Millennium Institute, The University of Sydney and Westmead Hospital, Westmead, NSW 2145, Australia
[b] Institute of Virology, University of Zurich, 8057 Zurich, Switzerland

ARTICLE INFO

Article history:
Received 23 June 2011
Returned to author for revision
22 October 2011
Accepted 1 November 2011
Available online 21 November 2011

Keywords:
pUL36
pUL37
Herpes simplex virus
Virus assembly
Tegument

ABSTRACT

The herpes simplex virus type 1 (HSV-1) structural tegument proteins pUL36 and pUL37 are essential for secondary envelopment during the egress of viral particles. For this study, scanning alanine mutagenesis of HSV-1 pUL37, in combination with yeast two-hybrid, identified pUL37 residue D631 as a major determinant for binding of pUL36. Further analysis of the binding of this pUL37 mutant to pUL36 by coimmunoprecipitation assay confirmed the role of pUL37 D631 in mediating binding of pUL36. A *trans*-complementation assay using pUL37 deletion virus FRΔUL37 was then carried out, where pUL37 wild type or D631A were provided in *trans*. For pUL37 D631A, a significant reduction in virus titer was observed compared to that seen when pUL37 wild type was present. The results presented here underline the crucial role of the pUL36/pUL37 interaction in replication of HSV-1 and indicate a critical role for pUL37 D631 in mediating this interaction.

Introduction

The herpesvirion is composed of four components: a double stranded DNA genome enclosed in an icosahedral capsid, an amorphous protein layer termed the tegument and a glycoprotein-containing host cell membrane-derived envelope (Roizman et al., 2007). The most widely accepted model for herpesviral assembly, based primarily on studies with the *Alphaherpesvirinae* subfamily members herpes simplex virus type 1 (HSV-1) and pseudorabies virus (PrV), is the primary envelopment–deenvelopment–secondary envelopment pathway (Mettenleiter et al., 2009). In this model nucleocapsids (capsid containing DNA genome) are assembled in the nucleus before undergoing primary envelopment by budding through the inner nuclear membrane into the perinuclear space. This primary envelope then fuses with the outer nuclear membrane and deenveloped nucleocapsids are deposited in the cytoplasm. Acquisition of inner tegument onto the nucleocapsid precedes secondary envelopment, acquisition of outer tegument and glycoprotein envelope, in or close to the Golgi. Fully assembled virions are finally released by exocytosis.

The tegument layer consists of around 20 viral-encoded proteins (Loret et al., 2008; Mettenleiter, 2004; Roizman et al., 2007) with both structural and non-structural functions (Kelly et al., 2009). During entry of a host cell inner tegument proteins pUS3, pUL36 and pUL37 have been shown to remain attached to the capsid while the remaining tegument and envelope are lost (Antinone and Smith, 2010; Copeland et al., 2009; Granzow et al., 2005). The inner tegument proteins pUL36 and pUL37, which are conserved across all members of the *Herpesviridae* family, are required for normal maturation of capsids into enveloped virions. For HSV-1 in cell lines, deletion of the genes encoding pUL36 or pUL37 blocks further addition of tegument to capsids in the cytoplasm (Desai, 2000; Desai et al., 2001; Leege et al., 2009; Roberts et al., 2009). For PrV, while pUL36 is essential for replication of the virus, pUL37 is not essential for productive infection in cell lines (Fuchs et al., 2004; Klupp et al., 2002; Leege et al., 2009). Initial addition of pUL36 to the capsid appears to be dependent on a direct interaction between the C-terminus of pUL36 and the capsid protein pUL25. This interaction has been identified in a number of *Alphaherpesvirinae* subfamily members including HSV-1 and PrV, using coimmunoprecipitation of transiently expressed pUL25 and pUL36 (Coller et al., 2007; Pasdeloup et al., 2009), and in varicella zoster virus (VZV), using yeast two-hybrid (Uetz et al., 2006). In the case of HSV-1, deletion of pUL36 has been shown to block subsequent addition of pUL37 to nucleocapsids (Ko et al., 2010). Addition of pUL37 is thought to be through a direct interaction with pUL36 which is conserved across the *Herpesviridae* family. The interaction of pUL36 with pUL37 has been identified using yeast two-hybrid and coimmunoprecipitation in HSV-1 (Lee et al., 2008; Vittone et al., 2005), PrV (Klupp et al., 2002), VZV (Stellberger et al., 2010; Uetz et al., 2006), human cytomegalovirus (HCMV) (Bechtel and Shenk, 2002; To et al., 2011) and Kaposi's sarcoma-associated herpesvirus (KSHV) (Rozen et al., 2008).

Observed localization of HSV-1 pUL37 to the Golgi in infected cells suggests this is the location where the protein, in complex with pUL36,

---

* Corresponding author at: Centre for Virus Research, Westmead Millennium Institute, PO Box 412, Westmead 2145 NSW, Australia. Fax: +61 2 9845 9103.
E-mail address: russell.diefenbach@sydney.edu.au (R.J. Diefenbach).

0042-6822/$ – see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.virol.2011.11.002

is added to cytoplasmic capsids (Desai et al., 2008). The observation that deletion of pUL36 in HSV-1 blocks trafficking of pUL37 to the Golgi in a capsid-independent manner (Desai et al., 2008), suggests the interaction between these two proteins is key for their incorporation into virions. Initial addition of pUL36 and pUL37 to HSV-1 nucleocapsids has been proposed to occur in the nucleus (Bucks et al., 2007). Other studies on both HSV-1 (Radtke et al., 2010; Trus et al., 2007; Wolfstein et al., 2006) and PrV (Mohl et al., 2009) indicate addition of pUL36 to the nucleocapsid occurs only in the cytoplasm.

The 273 kDa pUL36 contains a number of essential and nonessential domains (Mohl et al., 2010). It is required for intracellular nucleocapsid transport during egress (Luxton et al., 2006; Shanda and Wilson, 2008) and is a likely receptor for molecular motors (Radtke et al., 2010; Wolfstein et al., 2006). It also plays a role in release of viral DNA from the nucleocapsids during entry (Batterson and Roizman, 1983; Batterson et al., 1983; Delboy et al., 2008; Jovasevic et al., 2008; Knipe et al., 1979). A conserved deubiquitinating enzyme activity has been identified in the N-terminal region of pUL36 (Kattenhorn et al., 2005; Schlieker et al., 2005), with a nuclear localization signal found adjacent to it (Abaitua and O'Hare, 2008).

The 120 kDa pUL37 has been found to increase the efficiency of transport of nucleocapids to the nucleus, although unlike pUL36, it is not essential for this process (Krautwald et al., 2009; Luxton et al., 2006). It has been shown not to have a role in nuclear egress of either HSV-1 or PrV (Leege et al., 2009) but is required to direct nucleocapsids to the site of secondary envelopment at the TGN (Pasdeloup et al., 2010). It has been found to coelute with the single-stranded DNA binding protein ICP8 (Shelton et al., 1994), it activates NF-κB signaling via binding to TNF receptor-associated factor 6 (TRAF) (Liu et al., 2008), and possesses a nuclear export signal (NES) (Watanabe et al., 2000).

The pUL37-binding region in pUL36 has been previously defined for both HSV-1 and PrV to HSV-1 residues 512–608 (Klupp et al., 2002; Mijatov et al., 2007). We subsequently identified a number of residues in HSV-1 pUL36 within the described pUL37-binding region, including F593, E596 and to a lesser extent E580, which mediate interaction with pUL37 (Mijatov et al., 2007). The pUL37 region which binds pUL36 has yet to be fully characterized, although a recent study identified the carboxy terminal half of pUL37 as important for interaction with pUL36 (Bucks et al., 2011). In this study, we sought to define further the pUL36-binding region within HSV-1 pUL37. Single alanine substitutions were engineered into pUL37 and tested for binding against pUL36 using a yeast two-hybrid assay, allowing identification of a key conserved amino acid residue, D631, which is essential for binding to pUL36 *in vitro*. Further analysis by coimmunoprecipitation assay confirmed the role of pUL37 D631 in mediating binding of these two inner tegument proteins. When a *trans*-complementation assay was carried out, where wild type or mutant forms of pUL37 were tested for their ability to complement the pUL37 deletion virus FRΔUL37, mutation of the pUL37 D631 residue was found to significantly reduce production of infectious virus. The results presented here offer further insight into the highly conserved interaction between inner tegument proteins pUL36 and pUL37 and provide details of a single amino acid residue of pUL37 which plays a key role in mediating this interaction.

Results

Alanine-scanning mutagenesis of pUL37

A preliminary attempt to define a pUL36-binding domain in pUL37 using a series of pUL37 truncations in the yeast two-hybrid assay was unsuccessful (not shown). This is in contrast to a recent study which used a coimmunoprecipitation assay rather than a yeast two-hybrid approach (Bucks et al., 2011). We then undertook scanning alanine substitution of conserved HSV-1 pUL37 residues. Alignment of HSV-1 pUL37 with sequences from several other members of the *Alphaherpesvirinae* subfamily identified a total of 110 conserved residues located throughout pUL37 (Fig. 1). As it was not feasible to mutate all residues, a total of seventeen of these pUL37 residues were chosen, predominantly based on charge, and targeted for site-directed mutagenesis in the hope of identifying key residue(s) which mediate an interaction with pUL36. The current study was initiated prior to the recent report identifying a minimal pUL36 binding domain in HSV-1 pUL37 corresponding to residues 568–1123 (Bucks et al., 2011). The pUL37 substitutions to alanine were generated in a background of Target/pUL37 full-length and assessed for their ability to bind Bait/pUL36(1–767) using the yeast two-hybrid assay (Fig. 2A). A liquid β-galactosidase assay indicated that the most significant reduction in binding of pUL36, in the range 1–26% of wild type, was observed for the substitutions W175A, F420A, Y480A and D631A (Fig. 2A). Of these, only the W175A mutation maps to a proposed pUL37 self-association domain while D631A maps to a recently identified pUL36 binding domain (Bucks et al., 2011). Intermediate reductions, in the range 43–74% of wild type, were observed for E108A, R117A, R265A, D274A, R530A, R553A, R657A and R671A (Fig. 2A). Substitutions which had the least effect, in the range 79–113%, of wild type included R158A, Q232A, R348A, D658A and D1038A (Fig. 2A). For each Target/pUL37 mutation no autoactivation was observed when tested against Bait/no insert (not shown). Each of the Target/pUL37 alanine substitutions was also confirmed to be expressed in yeast at equivalent levels to wild type (Fig. 2B).

Coimmunoprecipitation assay

The four pUL37 mutants (W175A, F420A, Y480A and D631A) which exhibited the most significant reduction in binding to pUL36 by yeast two-hybrid analysis were further analyzed by coimmunoprecipitation assay. Both wild type pUL37 and pUL37 alanine mutant coding sequences were cloned into the pCMVmyc mammalian expression vector. The coding sequence of a further pUL37 mutant (R158A) which did not affect binding of pUL37 to pUL36 according to yeast two-hybrid analysis, was also cloned into the pCMVmyc vector to serve as a control to ensure that any effect observed for the alanine mutants was specific to those particular amino acid changes. The region of pUL36 encoding the pUL37 binding site (fragment N2; amino acids 512–767) was cloned into pEGFP-N1 vector and expressed as a GFP fusion protein. A region of pUL36 adjacent to the pUL37 binding site (fragment N1; amino acids 317–511) was also expressed as a GFP fusion protein, and served as a negative control.

Hela cells were cotransfected with expression constructs encoding pUL36N1-GFP or pUL36N2-GFP and each of the myc-pUL37 expression constructs encoding myc-pUL37WT, myc-pUL37R158A, myc-pUL37W175A, myc-pUL37F420A, myc-pUL37Y480A, or myc-pUL37D631A. Cell lysates were harvested at 24 h post transfection and coimmunoprecipitation with anti-myc antibody was carried out. Western blot of input lysates confirmed expression of myc and GFP fusion proteins (Fig. 3A). Analysis of coimmunoprecipitation samples by western blot using an anti-GFP antibody revealed that pUL36N1-GFP was not precipitated by any of the myc-tagged pUL37 proteins (Fig. 3B). In samples coexpressing pUL36N2-GFP and myc-tagged pUL37 proteins, pUL36N2-GFP precipitated with myc-pUL37WT and myc-pUL37R158A, as expected. Despite lower levels of myc-pUL37W175A, myc-pUL37F420A and myc-pUL37Y480A in the lysates (Fig. 3A) and coimmunoprecipitation samples (Fig. 3B), each of these mutants also precipitated pUL36N2-GFP, albeit at a correspondingly lower level compared to myc-pUL37WT (Fig. 3B). The level of myc-pUL37D631A detected in the coimmunoprecipitation sample was similar to that of myc-pUL37WT (1.4 fold that of the wild type) (Fig. 3B), however the amount of pUL36N2-GFP that precipitated with myc-pUL37D631A was 16-fold less than with myc-pUL37WT (Fig. 3B). A 7 fold lower level of pUL36N2-GFP expression was detected in samples coexpressing myc-pUL37D631A compared to those coexpressing myc-