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Identification of structural protein–protein interactions of herpes simplex virus type 1

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ABSTRACT

In this study we have defined protein–protein interactions between the structural proteins of herpes simplex virus type 1 (HSV-1) using a LexA yeast two-hybrid system. The majority of the capsid, tegument and envelope proteins of HSV-1 were screened in a matrix approach. A total of 40 binary interactions were detected including 9 out of 10 previously identified tegument–tegument interactions (Vittone, V., Diefenbach, E., Triffett, D., Douglas, M.W., Cunningham, A.L., and Diefenbach, R.J., 2005. Determination of interactions between tegument proteins of herpes simplex virus type 1. J. Virol. 79, 9566–9571). A total of 12 interactions involving the capsid protein pUL35 (VP26) and 11 interactions involving the tegument protein pUL46 (VP11/12) were identified. The most significant novel interactions detected in this study, which are likely to play a role in viral assembly, include pUL35–pUL37 (capsid–tegument), pUL46–pUL37 (tegument–tegument) and pUL49 (VP22)–pUS9 (tegument–envelope). This information will provide further insights into the pathways of HSV-1 assembly and the identified interactions are potential targets for new antiviral drugs.

Introduction

The herpes simplex virion consists of four components that include a double stranded DNA genome (152kb), capsid, tegument, and an outer envelope (Roizman and Sears, 1996). The icosadeltahedral capsid encloses the DNA genome and consists of 6 external proteins pUL6, pUL18 (VP23), pUL19 (VP5), pUL25, pUL35 (VP26) and pUL38 (VP19C) (Roizman and Sears, 1996). The surrounding tegument contains 22 proteins that include pUL4, pUL11, pUL13, pUL14, pUL16, pUL17, pUL21, pUL36 (VP1/2), pUL37, pUL41, pUL46 (VP11/12), pUL47 (VP13/14), pUL48 (VP16), pUL49 (VP22), pUL51, pUL56, pUS2, pUS3, pUS10, pUS11, ICP0, and ICP4 (Mettenleiter, 2004). The envelope contains the 5 proteins, pUL20, pUL43, pUL45, pUL49A and pUS9 along with the 11 glycoproteins gpUL1 (gL), gpUL10 (gM), gpUL22 (gH), gpUL27 (gB), gpUL44 (gC), gpUL53 (gK), gpUS4 (gG), gpUS5 (gJ), gpUS6 (gD), gpUS7 (gI) and gpUS8 (gE) (Mettenleiter, 2004).

Two pathways have been proposed for viral assembly and egress from the host cell. The first model for viral assembly and egress involves the pathway of primary envelopment, de-envelopment and secondary envelopment (Mettenleiter, 2002, 2004, 2006). After assembly in the nucleus, the mature herpes simplex virus type 1 (HSV-1) nucleocapsid (capsid containing DNA genome) undergoes primary envelopment through the inner nuclear membrane into the perinuclear space. The enveloped particles are de-enveloped at the outer nuclear membrane. Acquisition of inner tegument (pUL36, pUL37 and pUS3) onto the capsid (which may possibly begin in the nucleus (Bucks et al., 2007)) precedes secondary envelopment, and acquisition of outer tegument (consisting primarily of pUL46, pUL47, pUL48 and pUL49) and glycoprotein envelope, in or close to the Golgi. The outer tegument and glycoprotein self-assemble within the cytoplasm in a process that is independent from the addition of the inner tegument to the capsid. Interaction between the capsid and capsidless tegument structures appears to be essential to drive secondary envelopment of the virus (Mettenleiter, 2006). Fully assembled virions are finally released by exocytosis. A second pathway for HSV assembly and egress has been recently proposed, suggesting that nucleocapsids exit directly into the cytoplasm *via* damaged nuclear pores before addition of tegument in the cytoplasm and envelopment at the Golgi (Leuzinger et al., 2005; Wild et al., 2005). This second pathway remains to be confirmed by other laboratories.

The use of the yeast two-hybrid assay to define viral protein interaction networks or interactomes (Diefenbach et al., 2005) has been successfully undertaken for a number of herpesviruses including the alphaherpesviruses HSV-1 (Vittone et al., 2005) and Varicella zoster virus (VZV) (Uetz et al., 2006), the gammaherpesvirus Epstein–Barr virus (Calderwood et al., 2007) and Kaposi's sarcoma-associated virus (Uetz et al., 2006; Rozen et al., 2008). Knowledge of the viral interactome, particularly amongst structural virion proteins, coupled with viral gene deletion and morphological studies has led to an emerging picture of the protein–protein interactions important for assembly and egress of HSV-1 and the related alphaherpesvirus Pseudorabies virus (PrV) (Vittone et al., 2005; Mettenleiter, 2006).

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In this study we have undertaken a yeast two-hybrid matrix screen using a library of the majority of the HSV-1 structural proteins from the capsid, tegument and envelope. This represents an extension of a partial screen using a library of 13 HSV-1 tegument proteins (Vittone et al., 2005). A total of 31 new interactions were detected.

Results

Yeast two-hybrid analysis of the interaction of HSV-1 structural proteins

In this study we have cloned and expressed, in the LexA yeast two-hybrid system, 6 of the external capsid proteins (pUL6, pUL18, pUL19, pUL25, pUL35 and pUL38), 20 of the 22 tegument proteins (pUL4, pUL11, pUL13, pUL14, pUL16, pUL17, pUL21, pUL36, pUL37, pUL41, pUL46, pUL47, pUL48, pUL49, pUL51, pUL56, pUS2, pUS3, pUS10 and pUS11) and 8 of the 16 envelope proteins (gM, gB, gC, pUL45, gD, gI, gE and also pUS9) of HSV-1 (Table 1). All were cloned into both Bait and Target vectors with the exception of pUL45 which could only be cloned into Target vector (Table 1). Proteins ICP0 and ICP4 were not included in the screen as they are minor components of the tegument and probably perform purely regulatory rather than structural roles (Yao and Courtney, 1989, 1992). The library of HSV-1 proteins were all full-length constructs with the following exceptions: pUL19 upper domain (UD) (aa 451–1054) (Bowman et al., 2003), pUL36N (aa 1–1874) (Douglas et al., 2004), pUL36N1 (aa 1–767) (Vittone et al., 2005), and the cytoplasmic tail of each envelope protein (Table 1). Full-length pUL19 was previously found to not express in the yeast two-hybrid assay (Douglas et al., 2004). The pUL19 UD contains the binding site for pUL35 and in the context of the capsid structure is accessible for interactions with other viral proteins (Bowman et al., 2003; Baker et al., 2006). The complete gene (9495bp) encoding the large tegument protein pUL36 could not be previously amplified in our laboratory (Douglas et al., 2004). As such we have focused on the N-terminal two thirds of the protein, as the majority of the C-terminus, except for the extreme C-terminal end, is dispensable for viral function, as shown for PrV (Bottcher et al., 2006; Lee et al., 2006; Bottcher et al., 2007; Coller et al., 2007). For ease of cloning the single transmembrane spanning envelope proteins were the focus of this study. Furthermore, the cytoplasmic tails were chosen as they have been postulated to form the link with the outer tegument proteins during secondary envelopment (Mettenleiter et al., 2006). A similar approach using the LexA yeast two-hybrid assay and the cytoplasmic tails of PrV glycoproteins has been previously described (Fuchs et al., 2002). In addition, the nature of the LexA yeast two-hybrid assay requires that interacting proteins translocate to the nucleus for expression of the nutritional reporter genes (Fashena et al., 2000). This is less likely if one of the protein partners contains a transmembrane domain which restricts it to the cytoplasm.

A yeast two-hybrid matrix screen (Diefenbach et al., 2005) testing all possible binary combinations was then undertaken using the library of HSV-1 structural proteins. A total of 40 interactions were initially identified using a qualitative plate-based assay (Table 2). These interactions included 6 capsid–capsid, 11 capsid–tegument, 5

Table 1
Summary of HSV-1 structural proteins tested in the yeast two-hybrid assay

Structural HSV-1 protein	Gene size (bp)	Mwt (Da)	Virion location	Essential in cell culture	Yeast two-hybrid assay			
					Fragment expressed (aa number)	Fragment size (Da)	Bait fusion size (Da)[a]	Target fusion size (Da)[a]
pUL6	2031	74091	Capsid	Yes	FL		96725	85924
pUL18 (VP23)	957	34271	Capsid	Yes	FL		56904	46104
pUL19 (VP5)	4125	149084	Capsid	Yes	451–1054	65103	87737	76936
pUL25	1743	62670	Capsid	Yes	FL		AA	74503
pUL35 (VP26)	339	12094	Capsid	No	FL		34728	23927
pUL38 (VP19C)	1398	50263	Capsid	Yes	FL		AA	62096
pUL4	600	21517	Tegument	No	FL		44151	33350
pUL11	291	10485	Tegument	No	FL		33119	22318
pUL13	1557	57196	Tegument	No	FL		79830 (NE)	69029
pUL14	660	23933	Tegument	No	FL		AA	35766
pUL16	1122	40442	Tegument	No	FL		AA	52275
pUL17	2112	74582	Tegument	Yes	FL		AA	86415
pUL21	1608	57641	Tegument	No	FL		AA	69474
pUL36 (VP1/2)	9495	335821	Tegument	Yes	1–1874, 1–767	199564, 81008	222198	92839
pUL37	3372	129574	Tegument	Yes	FL		143208	132407
pUL41	1470	54918	Tegument	No	FL		77552	66751
pUL46 (VP11/12)	2157	78244	Tegument	No	FL		100878	90077
pUL47 (VP13/14)	2082	73791	Tegument	No	FL		96425	85624
pUL48 (VP16)	1473	54345	Tegument	Yes	1–411, FL	68690	AA	66178
pUL49 (VP22)	906	32254	Tegument	No	FL		54888	44087
pUL51	735	25470	Tegument	No	FL		48104	37303
pUL56	705	25320	Tegument	No	FL		47954	37153
pUS2	876	32470	Tegument	No	FL		55104	44303
pUS3	1446	52834	Tegument	No	FL		AA	64667
pUS10	939	34055	Tegument	No	FL		AA	45888
pUS11	486	16692	Tegument	No	FL		39326	28525
gpUL10 (gM)	1442	51393	Envelope	No	343–473	15225	AA	27058
gpUL27 (gB)	2715	100292	Envelope	Yes	796–904	12281	34915	24114
gpUL44 (gC)	1536	54998	Envelope	No	501–511	1467	24101	13300 (NE)
pUL45	519	18233	Envelope	No	49–172	13334		25167
gpUS6 (gD)	1185	43347	Envelope	Yes	364–394	3839	26473	15672 (NE)
gpUS7 (gI)	1173	41369	Envelope	No	296–390	10279	32913	22112
gpUS8 (gE)	1653	59093	Envelope	No	447–550	11561	AA	23394 (NE)
pUS9	273	10026	Envelope	No	1–64	7341	29975	19174 (NE)

NE, not expressed; AA, autoactivates; FL, full-length.
[a] Bait only size 22,634 Da. Target only size 11,833 Da.

Table 2
Yeast two-hybrid matrix screen for binary interactions of HSV-1 structural proteins[a]

	Capsid						Tegument																		Envelope							
Target \ Bait	pUL6	pUL18	pUL19 UD	pUL25	pUL35	pUL38	pUL4	pUL11	pUL13	pUL14	pUL16	pUL17	pUL21	pUL36 N1	pUL37	pUL41	pUL46	pUL47	pUL48	pUL49	pUL51	pUL56	pUS2	pUS3	pUS10	pUS11	gM CT	gB CT	gC CT	pUL45 CT	gD CT	gI CT	gE CT	pUS9 CT
Capsid																																	
pUL6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	NE	-	NE	-	NE	NE
pUL18 (VP23)	-	+	-	-	-	+++	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	NE	-	NE	-	NE	NE
pUL19 UD (VP5)	-	-	-	-	-	-	-	-	-	-	++	-	+++	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	NE	-	NE	-	NE	NE
pUL25	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA
pUL35 (VP26)	-	+++	+	+	-	++	-	+	-	++	++	-	++	-	+++	-	-	-	++	-	+++	-	-	+	-	-	-	-	NE	-	NE	-	NE	NE
pUL38 (VP19C)	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA
Tegument																																	
pUL4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	NE	-	NE	-	NE	NE
pUL11	-	-	-	-	-	-	-	-	-	-	+++	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	NE	-	NE	-	NE	NE
pUL13	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE
pUL14	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA
pUL16	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA
pUL17	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA
pUL21	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA
pUL36 N (VP1/2)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+++	-	-	-	+++	-	-	-	-	-	-	-	-	-	NE	-	NE	-	NE	NE
pUL37	-	-	-	-	-	+	-	-	-	-	-	-	-	++	+++	-	-	-	-	-	-	-	-	-	-	-	-	-	NE	-	NE	-	NE	NE
pUL41	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	NE	-	NE	-	NE	NE
pUL46 (VP11/12)	-	+++	+	+	-	+	-	-	-	-	-	-	+	-	+++	-	-	-	+++	+	-	-	-	+++	+	-	++	-	NE	-	NE	-	NE	NE
pUL47 (VP13/14)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	NE	-	NE	-	NE	NE
pUL48–AD (VP16)	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA
pUL49 (VP22)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+++	+++	-	-	-	-	-	-	-	-	NE	-	NE	-	NE	NE
pUL51	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	NE	-	NE	-	NE	NE
pUL56	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	NE	-	NE	-	NE	NE
pUS2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	NE	-	NE	-	NE	NE
pUS3	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA
pUS10	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA
pUS11	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+++	-	-	NE	-	NE	-	NE	NE
Envelope																																	
gpUL10 CT (gM)	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	NE	AA	NE	AA	NE	NE
gpUL27 CT (gB)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+++	NE	-	NE	-	NE	NE
gpUL44 CT (gC)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	NE	-	NE	-	NE	NE
gpUS6 CT (gD)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	NE	-	NE	-	NE	NE
gpUS7 CT (gI)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	NE	-	NE	-	NE	NE
gpUS8 CT (gE)	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	NE	AA	NE	AA	NE	NE
pUS9 CT	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+++	-	-	-	-	-	-	-	-	NE	-	NE	-	NE	NE

Positive interactions (shaded) were observed as blue colonies with + indicating lightest blue and +++ the darkest blue. No growth (−) indicates a negative interaction.
AA, autoactivation; NE, not expressed; N, amino acids 1–1874; N1, amino acids 1–767; CT, cytoplasmic domain; −AD, minus activation domain (amino acids 412–490); UD, upper domain (amino acids 451–1054).
[a] Interactions were assessed using a qualitative in vivo plate assay (at day 3) with readout being expression of the reporter genes LEU2 and lacZ.

tegument–capsid, 15 tegument–tegument, 1 tegument–envelope, 1 envelope–tegument, and 1 envelope–envelope (Table 2). Within the tegument–tegument interactions 9 have been previously described in our laboratory (Vittone et al., 2005). Only the interaction of pUL36 with pUL37, was found in both orientations (Table 2). For pUL35 and pUL46 a high number of interactions were observed as baits but none as targets (Table 2). The influence of the fusion partner on correct folding of the fused viral protein may explain why the pUL35 and pUL46 interactions work in only one orientation. A high proportion (11) of bait constructs were found to autoactivate, which is a general observation of the yeast two-hybrid approach (Fashena et al., 2000), and hence could not be tested in the yeast two-hybrid screen (Table 1). In the case of pUL48, amino acids 1–411 (which lacked the C-terminal activation domain and referred to here as pUL48 − AD) were used as bait in an attempt to avoid previously observed autoactivation by full-length pUL48 (Douglas et al., 2004; Vittone et al., 2005). Amino acids 1–411 contain the core of pUL48 whose structure has been identified as containing regions sufficient for virion assembly (Liu et al., 1999). Unfortunately though strong autoactivation was still observed for Bait pUL48 − AD (Table 2). The majority of those HSV-1 proteins which did autoactivate as baits could at least be tested in the reverse orientation as a target fusion construct. The exception was the envelope protein gE which autoactivates as bait and is not expressed as target (Table 1). Autoactivation has been previously documented for PrV gE cytoplasmic tail (Fuchs et al., 2002). Indeed a large proportion of the envelope proteins either autoactivated as baits or were not expressed as targets which partly explains why so few interactions involving envelope proteins were identified (Table 1).

Quantitative β-galactosidase activity values were then obtained for each positive interaction using a liquid assay (Table 3). All values were less than the positive control of Bait p53/Target SV40 Large T-antigen (Table 3). For a number of interactions the β-galactosidase activity values were not significantly above the negative control of Bait no insert/Target no insert (Table 3). In most cases a reasonable correlation was found between the intensity of blue colour observed with the plate-based assay versus the β-galactosidase activity values obtained using a quantitative liquid assay (Table 3). Though there were some exceptions (Table 3). It should be noted that the quantitative liquid assay only depends on activation of the one reporter gene *lacZ* while the plate-based assay is more stringent as it depends on activation of two reporter genes *LEU2* and *lacZ*.

The expression of bait and target fusion constructs in the LexA yeast two-hybrid assay was assessed by immunoblotting for either the LexA tag (present in bait fusions) (Fig. 1) or the hemagglutinin (HA) tag (present in target fusions) (Fig. 2). This was to establish that any negative interaction was not the result of the absence of expression in the yeast two-hybrid assay. The large majority of fusion proteins were expressed with varying degrees of proteolysis (Figs. 1 and 2). Although a yeast protease inhibitor cocktail (Sigma product number P8215) was included during sample extraction the observed proteolysis most likely occurred during the extraction due to the range of strong endogenous proteases in yeast. Although a number of the bait proteins, particularly the tegument proteins pUL36N, pUL37, pUL41, pUL46, pUL47 and pUL51, appear to be expressed at low levels (Fig. 1), at least some of these, pUL36N, pUL37 and pUL46, were still able to produce positive interactions in the yeast two-hybrid screen (Table 2). These same tegument proteins were all well expressed as targets (Fig. 2). This provided the opportunity to establish interactions which may have not have been detected as baits due to the low expression levels. The only bait fusion that was not expressed was bait/pUL13 (not shown). A band for bait/pUL36N was not apparent in the immunoblots (based on a predicted size of ~200 kDa (Table 1) it most likely did not migrate into the 12% gel) (Fig. 1) but interactions were observed with this construct (Table 2). In the case of target fusions the cytoplasmic tails of gC, gD, gE and pUS9 were not expressed (Fig. 2).

Table 3
Summary of quantitative liquid β-galactosidase assay for yeast two-hybrid positive interactions

Interaction	β-galactosidase activity	Plate score
No insert/no insert (negative control)	6±2	–
p53/large T-antigen (positive control)	2274±258	3 plus
Capsid/capsid		
pUL18/pUL18	10±5	1 plus
pUL18/pUL38	108±28	3 plus
pUL35/pUL18	1422±357	3 plus
pUL35/pUL19	531±48	1 plus
pUL35/pUL25	390±119	1 plus
pUL35/pUL38	1510±203	2 plus
Capsid/tegument		
pUL19/pUL16	18±4	2 plus
pUL19/pUL21	264±16	3 plus
pUL19/pUL48	31±15	1 plus
pUL35/pUL11	12±2	1 plus
pUL35/pUL14	49±15	2 plus
pUL35/pUL16	9±2	2 plus
pUL35/pUL21	55±10	2 plus
pUL35/pUL37	1180±32	3 plus
pUL35/pUL48	172±24	2 plus
pUL35/pUL51	129±38	3 plus
pUL35/pUS3	59±2	1 plus
Tegument/capsid		
pUL37/pUL38	25±6	1 plus
pUL46/pUL18	1431±50	3 plus
pUL46/pUL19	22±9	1 plus
pUL46/pUL25	50±6	1 plus
pUL46/pUL38	50±6	1 plus
Tegument/tegument		
pUL4/pUL56	8±2	1 plus
pUL11/pUL16	1482±127	3 plus
pUL36/pUL37	135±28	3 plus
pUL36/pUL48	76±16	3 plus
pUL37/pUL36	87±12	2 plus
pUL37/pUL37	448±28	3 plus
pUL46/pUL21	3±2	1 plus
pUL46/pUL37	67±9	3 plus
pUL46/pUL48	79±23	3 plus
pUL46/pUL49	74±3	1 plus
pUL46/pUS3	25±3	3 plus
pUL46/pUS10	15±1	1 plus
pUL49/pUL48	1483±164	3 plus
pUL49/pUL49	147±17	3 plus
pUS11/pUS11	394±20	3 plus
Tegument/envelope		
pUL46/gM	162±17	2 plus
Envelope/tegument		
pUS9/pUL49	210±38	3 plus
Envelope/envelope		
gB/gB	263±44	3 plus

Discussion

In this study we have identified a number of binary interactions involving HSV-1 structural proteins using the LexA yeast two-hybrid matrix screen (Tables 1 and 2). Although the yeast two-hybrid approach is a relatively straightforward way to screen for large numbers of protein interactions, like any approach it does have some limitations (Diefenbach et al., 2005). Even related yeast two-hybrid

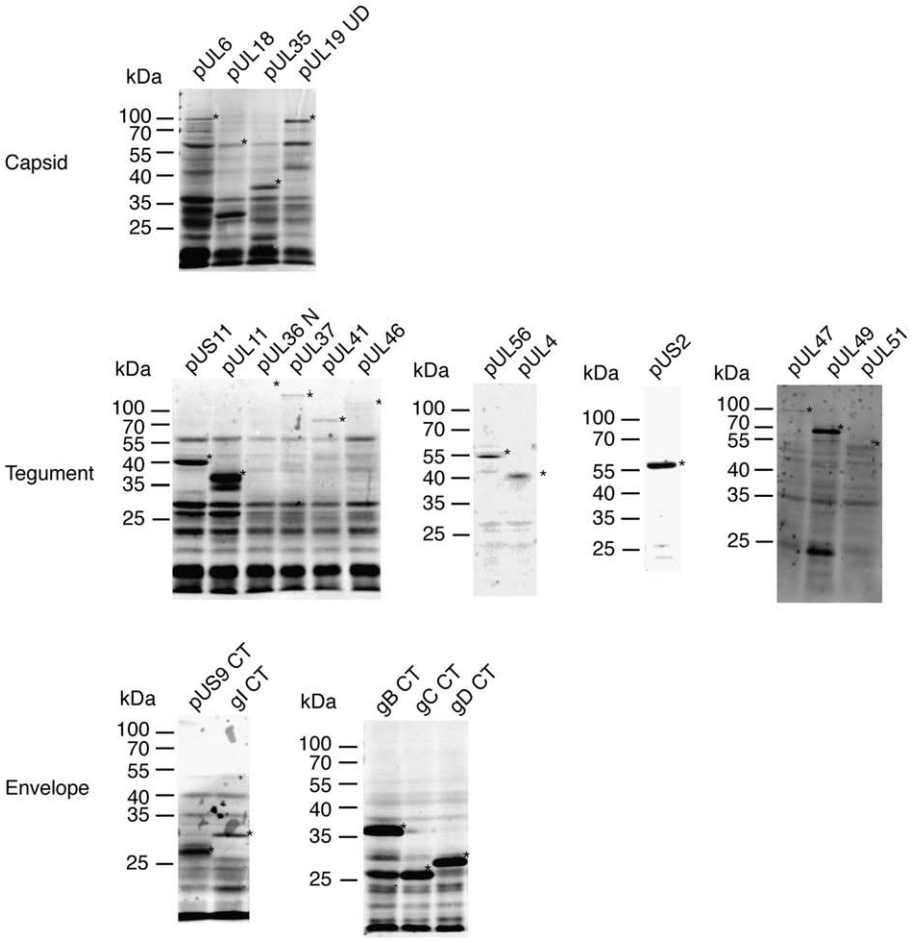

Fig. 1. Expression of bait/HSV-1 structural proteins in yeast. Yeast mated with various combinations of Bait and Target fusion constructs were subjected to protein extraction before separation by SDS-PAGE (12%). Immunoblot with anti-LexA showing expression of Bait fusion constructs. The positions of the fusion protein based on predicted molecular weights (Table 1) are indicated (*). The common bands observed in each lane reflect non-specific detection by the primary antibody as observed with Bait only (not shown). The specific multiple bands observed in most lanes are due to proteolysis. UD, upper domain of pUL19; N, aa 1–1874; CT, cytoplasmic tail.

approaches do not usually give rise to the same results (Uetz et al., 2006). This study though does represent one of the few proteomics-based approaches to the study of viral structural biology and only the second such approach for an alphaherpesvirus, the other being for VZV (Uetz et al., 2006). Whether the identified interactions are structural, regulatory or other requires further elucidation. However, from the work of others and the current model for viral assembly (Mettenleiter et al., 2006; Mettenleiter, 2006) a number of the observed interactions are likely to be important for assembly.

Capsid–capsid interactions

Of the 6 capsid–capsid interactions identified (Table 2), 3 have been previously identified. This includes the interaction of the triplex-forming capsid proteins pUL18 and pUL38, which plays an essential role in capsid assembly and architecture (Desai and Person, 1996; Zhou et al., 2000; Adamson et al., 2006; Okoye et al., 2006). The self-association of pUL18 has been previously described and appears to be an intermediate in the formation of capsid triplexes (consist of 1 copy of pUL38 and 2 copies of pUL18) (Spencer et al., 1998). The interaction between pUL19 UD and pUL35 has also been documented, though this interaction is not essential for capsid assembly (Chen et al., 2001; Bowman et al., 2003; Baker et al., 2006). Novel interactions identified in this study include pUL35–pUL18, pUL35–pUL25 and pUL35–pUL38. The precise role of these interactions in capsid assembly requires further investigation. In the case of pUL35–pUL18 and pUL35–pUL38 they are unlikely to be directly involved given the architecture of the capsid (Zhou et al., 2000). In addition, these interactions were not identified in a previous investigation of the pairwise interactions required for transport of capsid assembly intermediates into the nucleus, the final site of capsid assembly (Rixon et al., 1996). Nevertheless, the possibility still exists that these interactions may contribute to nuclear localization of one or both of the interacting partners.

Capsid–tegument interactions

A total of 16 interactions were identified involving capsid and tegument proteins (Table 2). Of these interactions half involved the capsid protein pUL35. The relative importance of these interactions in viral assembly needs further investigation. Deletion of pUL35 appears to not have any significant effect on tegumentation of the capsid (Chen et al., 2001). In addition, deletion of pUL35 has no significant effect on viral replication in cell lines but does appear to play a role in virion production when tested in mouse ganglia (Desai et al., 1998). Only 2 of the identified capsid–tegument interactions have been documented elsewhere. Interaction of HSV-2 capsid protein pUL35 with tegument proteins pUL14 has been suggested on the basis of a cellular relocalization assay and as such likely plays a non-structural role (Yamauchi et al., 2001). Interaction of capsid protein pUL35 with tegument protein pUL37 has also been reported for VZV (Uetz et al., 2006). The interaction of both the tegument proteins pUL37 and pUL48 with pUL35 may provide additional attachment of the tegument to the capsid *via* the hexons. Additional tegument–capsid

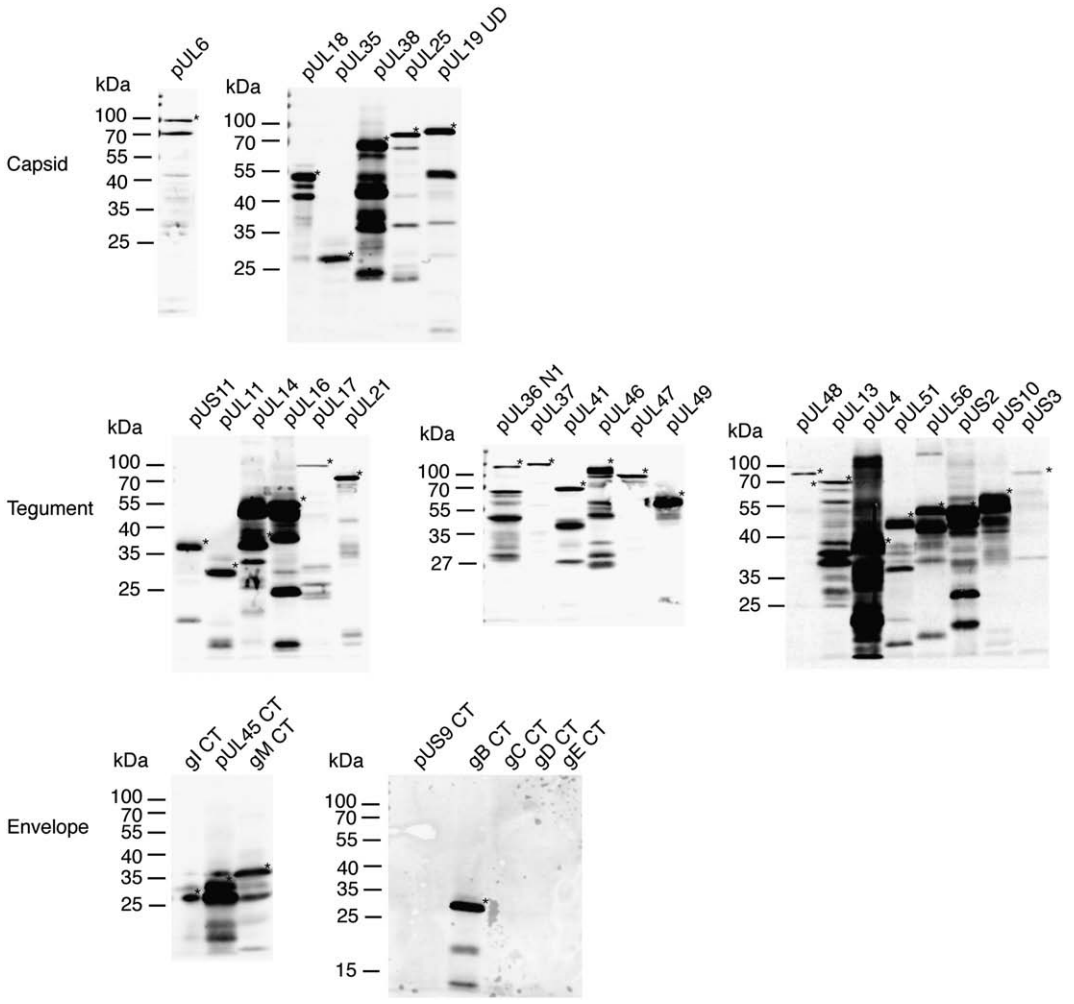

Fig. 2. Expression of target/HSV-1 structural proteins in yeast. Yeast mated with various combinations of Bait and Target fusion constructs were subjected to protein extraction before separation by SDS-PAGE (12%). Immunoblot with anti-HA showing expression of Target fusion constructs. The positions of the fusion protein based on predicted molecular weights (Table 1) are indicated (*). The specific multiple bands observed in most lanes are due to proteolysis. UD, upper domain of pUL19; N1, aa 1–767; CT, cytoplasmic tail.

interactions may be mediated by the observed interactions of pUL46 with pUL18 and pUL21 with pUL19. The reported complex of the capsid protein pUL25 and the tegument protein pUL17 (Trus et al., 2007) could not be assessed in this study due to autoactivation by both proteins as baits (Table 1). Another reported interaction of the capsid protein pUL25 and the tegument protein pUL36 in PrV (Coller et al., 2007) and VZV (Uetz et al., 2006) could not be verified in this study. This is because the interaction depends on the C-terminus of pUL36 (Coller et al., 2007) which was absent from the pUL36 fragments tested in the current yeast two-hybrid screen.

Tegument–tegument interactions

A total of 15 interactions between tegument proteins were identified (Table 2). Of these interactions, 9 were identified previously in our laboratory (Vittone et al., 2005). These included pUL11–pUL16, pUL36–pUL37, pUL36–pUL48, pUL37–pUL36, pUL37–pUL37, pUL46–pUL48, pUL49–pUL48, pUL49–pUL49 and pUS11–pUS11. The only interaction not confirmed was that of pUL47 and pUL48 which in our original screen was one of the weakest interactions (Vittone et al., 2005). It may not have been detected in this current study as the interactions were determined in diploid yeast, a result of yeast mating, rather than haploid yeast. As these interactions have been discussed previously (Vittone et al., 2005) we will only touch on the pUL36–pUL37 interaction here. This interaction between two essential tegument proteins has now been confirmed in a number of herpesviruses including HSV-1 (Vittone et al., 2005), PrV (Klupp et al., 2002), VZV (Uetz et al., 2006) and Kaposi's Sarcoma-associated virus (Rozen et al., 2008). For HSV-1, the interaction has also been further defined at the amino acid level (Mijatov et al., 2007) and although deletion of the pUL37-binding site in PrV pUL36 does not completely block viral assembly (Fuchs et al., 2004) it remains to be determined whether the same phenotype occurs in HSV-1. The study with PrV did not show whether blockage of pUL37 binding to pUL36 leads to loss of pUL37 in assembled virions. As indicated above the observed interaction of pUL35 with pUL37 may provide an alternative contact for binding of pUL37 to the capsid, rather then *via* pUL36 which itself interacts with pUL19 (McNabb and Courtney, 1992; Baker et al., 2006). An interaction was found between pUL46 and pUL37, at least in one orientation (Table 2), which was not detected in our previous study (Vittone et al., 2005). This interaction may be of significance in providing a link between the inner and outer tegument layers of HSV-1. An interaction was identified between the protein kinase pUS3 and pUL46. The reported viral protein substrates for HSV-1 pUS3 do not include pUL46 (Kato et al., 2005). Whether this is a structural or regulatory interaction requires further investigation.

Tegument–envelope and envelope–envelope interactions

A total of 3 new interactions were identified involving the cytoplasmic tails of envelope proteins (Table 2). These included pUL46–gM and pUS9–pUL49. These tegument–envelope interactions

are likely to be involved in the process of secondary envelopment during viral assembly. A number of interactions, not detected in this study, that likely play a role in secondary envelopment have been previously reported between tegument proteins and cytoplasmic tails of herpesviral glycoproteins. These include pUL48 with gH (Gross et al., 2003), and pUL49 with gE/gI, gM and gD (Fuchs et al., 2002; Farnsworth et al., 2007; O'Regan et al., 2007). The reasons these interactions were not detected include the absence of gH from the current screen, autoactivation or lack of expression of gE resulting in exclusion from the current screen. In addition, expression of the cytoplasmic tails of the envelope proteins alone would not have detected interactions that depend on oligomerization *via* the envelope protein's transmembrane domains or prior complex formation between different envelope proteins. One self-association was observed between the cytoplasmic tails of gB (Table 2). The precise role of such an interaction is not clear. The dimerization of gB has been previously reported and although it did not require the cytoplasmic tail the possible dimerization of the tail alone was never directly assessed (Laquerre et al., 1996).

Knowledge of HSV-1 structural protein–protein interactions will provide insights into the pathways of viral assembly and the identified interactions will permit the design and/or identification of candidate antiviral agents for treatment of neurotropic virus infections, particularly alphaherpesvirus infections. Our strategy to target sites of viral structural protein assembly provides an alternative to the current focus of drug development which is based on identifying inhibitors of specific HSV-1 enzymes such as DNA polymerase, ribonucleotide reductase and proteases (Naesens and De Clercq, 2001). The use of complementary approaches to confirm the yeast two-hybrid interactions and to look for higher order complexes (yeast two-hybrid assay only detects binary interactions) is currently underway. To do so we are in the process of generating HSV-1 constructs with affinity tags on individual key viral proteins such as pUL36, pUL37 or pUL48. These tags will be exploited to isolate protein complexes, dependent on the tagged protein, under native conditions from infected cells at various time points post-infection. Information on higher order protein complexes would not only shed light on intermediate complexes formed during viral assembly but may also reveal the role of cellular proteins in this process.

Materials and methods

Expression constructs

The cloning of HSV-1 capsid genes (*UL6*, *UL18*, *UL25*, *UL35* and *UL38*) and tegument genes (*UL11*, *UL14*, *UL16*, *UL17*, *UL21*, *UL36*, *UL37*, *UL41*, *UL46*, *UL48*, *UL49* and *US11*) into the yeast two-hybrid vectors displayBait and displayTarget (Display Systems Biotech) has been previously described (Douglas et al., 2004; Vittone et al., 2005). Additional HSV-1 genes cloned in this study included the capsid gene *UL19*, the 7 tegument genes *UL4*, *UL13*, *UL51*, *UL56*, *US2*, *US3* and *US10* and the 8 envelope genes *gM*, *gB*, *gC*, *UL45*, *gD*, *gI*, *gE* and *US9*. In the case of each envelope gene only the cytoplasmic tail was cloned (Table 1). Each viral gene was amplified from a HSV-1 strain 17 cosmid library (kindly provided by Charles Cunningham, MRC, Glasgow) (Cunningham and Davison, 1993) before insertion into either EcoRI digested (for *UL51* and *US3*) or EcoRI/XhoI digested (for the reminder) displayBait or displayTarget, as previously described (Douglas et al., 2004; Vittone et al., 2005). Gateway™ cloning technology (Invitrogen) was used for the region of *UL19* gene corresponding to the upper domain (amino acids 451–1054) (Bowman et al., 2003). A CACC motif was engineered into the 5′ end of the forward primer, to enable base pairing with the GTGG overhang in the entry vector pENTR™/D-TOPO® vector (Invitrogen). The *UL19* gateway entry clone was then used to introduce the *UL19* upper domain insert into either displayBait or displayTarget that had been made compatible with the gateway system using a Gateway™ vector conversion system (Invitrogen).

Yeast two-hybrid assay

The use of the LexA-based yeast two-hybrid assay has been previously described (Douglas et al., 2004; Vittone et al., 2005). However, to facilitate high throughput the yeast two-hybrid assay was modified to incorporate yeast mating. Briefly, two mating strains of haploid yeast were used. The *Saccharomyces cerevisiae MATα* strain EGY48 (Clontech) was transformed with both the displayBait construct and the reporter plasmid pSH18–34 (Invitrogen) whereas the *S. cerevisiae MATa* strain YM4271 (Clontech) was transformed with the displayTarget construct. Single transformants were subsequently picked from appropriate selection agar plates and resuspended in 1 ml of liquid YPD. Then 20 µl of the cell suspension for each haploid strain was aliquoted in the same well of a 96 well plate containing 160 µl of liquid YPD. The 96 well plate was incubated at 30 °C for 18 h to allow enough time for the haploid cells to fuse and become diploid cells containing displayBait and displayTarget constructs as well as the reporter plasmid. After incubation, 100 µl of diploid yeast cells were plated out on master agar plates. The protocols for qualitative assessment of protein–protein interactions, quantification of each positive interaction using a β-galactosidase assay and determination of protein expression in yeast were then as previously described (Douglas et al., 2004; Vittone et al., 2005). The β-galactosidase activity was calculated from the following equation: β-galactosidase activity = $1000 \times A_{420}/(t \times V \times OD_{660})$ where t = time (min) of incubation and V = volume of cells (ml) used in the assay. The values obtained for β-galactosidase activity were the average of measurements from three separate colonies.

Analysis of protein expression

Proteins were separated by SDS-PAGE and for analysis of expression in yeast were processed as previously described (Diefenbach et al., 1998; Vittone et al., 2005). Detection was performed with an Odyssey Infra-red Imaging system (Licor-Biosciences). Primary antibodies used included mouse monoclonal antibody against HA (1:500 dilution; Santa Cruz Biotechnology) and rabbit polyclonal against LexA (1:500 dilution; Sigma). Secondary antibodies for the Odyssey system included goat anti-mouse Alexa Fluor 680-conjugated IgG (used at 1:5000 dilution; Molecular Probes) and goat anti-rabbit IRdye800-conjugated IgG (used at 1:5000 dilution; Rockland Inc.).

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