# The human DnaJ protein, hTid-1, enhances binding of a multimer of the herpes simplex virus type 1 UL9 protein to $ori_S$, an origin of viral DNA replication

**Chi-Yong Eom and I. Robert Lehman***

Department of Biochemistry, Beckman Center, Stanford University School of Medicine, Stanford, CA 94305-5307

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**We have identified cellular proteins that interact with the herpes simplex virus type 1 (HSV-1) origin-binding protein (UL9 protein) by screening a HeLa cell complementary DNA library by using the yeast two-hybrid system. Approximately $7 \times 10^5$ colonies were screened. Five of the 48 positive clones contained cDNAs that encoded the $p150^{Glued}$ component of the dynactin complex, three contained cDNAs for the neural F Box 42-kDa protein (NFB42), which is highly enriched in neural tissue, and three contained hTid-1, a human homologue of the bacterial DnaJ protein. We have focused in this report on the interaction of the viral UL9 protein with the cellular hTid-1. *In vitro* immunoprecipitation experiments confirmed that hTid-1 interacts with the UL9 protein. Electrophoretic mobility-shift assays indicated that the hTid-1 enhances the binding of UL9 protein to an HSV-1 origin, $ori_S$, and facilitates formation of the multimer from the dimeric UL9 protein. hTid-1 had no effect on the DNA-dependent ATPase or helicase activities associated with the UL9 protein. These findings implicate hTid-1 in HSV-1 DNA replication, and suggest that this cellular protein may provide a chaperone function analogous to the DnaJ protein in *Escherichia coli* DNA replication.**

The linear 152-kbp herpes simplex virus type 1 (HSV-1) genome encodes seven proteins that are essential for the replication of its DNA. These proteins comprise an origin-binding protein, OBP or UL9 protein (the product of the *UL9* gene), a DNA polymerase (*UL30*), a DNA polymerase processivity factor (*UL42*), a heterotrimeric helicase/primase complex (*UL5, UL52,* and *UL8*), and a single-strand-DNA-binding protein referred to as ICP8 [(*UL29*); refs. 1 and 2]. *In vivo* studies of HSV-1 DNA replication have shown that the linear genome circularizes shortly after infection of host cells, and that DNA replication proceeds by a rolling-circle (3, 4). However, the existence of three origins of replication (two copies of $ori_S$ and one copy of $ori_L$) and a sequence-specific origin binding protein make it likely that origin-dependent, theta-type DNA replication also occurs. Attempts to reconstitute origin-dependent HSV-1 DNA replication with the seven purified viral proteins have been unsuccessful, suggesting that other factors, possibly cellular proteins, are required (1, 2).

The UL9 protein very likely functions as a replication initiator. It can bind to and unwind the origins of HSV-1 DNA replication and may serve to recruit the DNA replication machinery to the origin (2, 5, 6). In this respect, the UL9 protein resembles the large tumor antigens (Tags) of simian virus 40 (SV40), polyoma virus, and the E1 protein of human papilloma virus [(HPV); refs. 7–9], both of which have been shown to initiate DNA replication at their respective origins.

The UL9 protein exists in solution as a homodimer of 94-kDa subunits and binds tightly and cooperatively to specific sequences within $ori_S$, designated Boxes I, II, and III. The UL9 protein also exhibits DNA-dependent ATPase and 3′ to 5′ helicase activity, both of which are enhanced by ICP8 (6, 10–12). Dnase I footprinting and potassium permanganate sensitivity analyses have demonstrated that protein–protein interaction between $ori_S$-bound UL9 homodimers leads to local DNA bending of the intervening AT-rich sequence and unwinding by the UL9 protein–ICP8 complex (5, 13, 14). The UL9 protein may also have a role as a docking protein, based on its interaction with the other replication proteins encoded by the *UL8*, *UL42*, and *UL29* genes (15–17).

To identify cellular proteins that interact with the UL9 protein, we used a yeast two-hybrid system to screen a HeLa cell cDNA library with the UL9 protein as bait. This approach led to the identification of a number of cDNA clones that interact with the UL9 protein, including the $p150^{Glued}$ component of the dynactin complex (18), the neural F Box 42-kDa protein (NFB42), both of which are enriched in the nervous system (19), and hTid-1, a human homologue of the bacterial DnaJ protein (20).

We report here that the cellular hTid-1 protein interacts with viral UL9 protein *in vitro* and enhances binding of the UL9 protein to $ori_S$. This interaction facilitates the formation of the multimer from the dimeric form of the UL9 protein.

## Materials and Methods

**Yeast Two-Hybrid Screen.** Two-hybrid screening was carried out by using the Matchmaker Gal4 Two-Hybrid System 3 (CLONTECH). The full-length coding region of UL9 gene was amplified by PCR by using the appropriate synthetic primers and HSV-1 DNA, and inserted into the pGBKT7 vector to create the GAL4 DNA binding domain fusion construct. This plasmid, designated pGBKUL9, was used as bait to screen a human HeLa cDNA library that was cloned into the activation domain vector pACT2. The yeast reporter strain AH109 (*MATα*, *trp1–901*, *leu2–3*, *112*, *ura3–52*, *his3–200*, *gal4*Δ, *gal80*Δ, *LYS2*::$GAL1_{UAS}$-$GAL1_{TATA}$-$HIS_3$, $GAL2_{UAS}$-$GAL2_{TATA}$-*ADE2*, *URA3*::$MEL1_{UAS}$-$MEL1_{UAS}$-$MEL1_{TATA}$-*lacZ*) was transformed simultaneously with pGBKUL9 and the cDNA library by using the lithium acetate method according to the manufacturer's instructions. Transformants were selected by culture on minimal synthetic dropout medium lacking Ade, His, Leu, and Trp, and including 5-bromo-4-chloro-3-indolyl-α-D-galactoside for 5 days. To monitor the two-hybrid interactions of positive clones, β-galactosidase activity was estimated by the colony-lift filter assay according to the manufacturer's instructions. Plasmids from positive clones were isolated and analyzed by DNA sequencing.

**Nucleotide Sequence Analysis.** Automated sequencing was carried out to determine the nucleotide sequence of positive clones. DNA and protein sequence analyses were performed with the BLAST algorithm at the National Center for Biotechnology Information (NCBI).

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Abbreviations: HSV-1, herpes simplex virus type 1; SV40, simian virus 40; HPV, human papilloma virus; EMSA, electrophoretic mobility-shift assay; Tag, T-antigen; GST, glutathione *S*-transferase.

*To whom reprint requests should be addressed. E-mail: blehman@cmgm.stanford.edu.

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**Proteins and Oligonucleotides.** Recombinant UL9 protein and ICP8 were purified as previously described (11, 21). The Box I substrate was used for the DNA helicase assay (11). Polyclonal anti-UL9 antibody was prepared as described (21).

***In Vitro* Transcription and Translation of hTid-1.** Synthesis of full-length hTid-1 by *in vitro* transcription and translation of plasmid (pGEM-Tid1; ref. 20) carrying the full-length coding sequence of hTid-1 cloned into pGEM-7Zf(+) was carried out in a 50-$\mu$l reaction volume by using the TnT-coupled Reticulocyte Lysate System (Promega) in the presence of [$^{35}$S]L-methionine (New England Nuclear) according to the manufacturer's instructions. Newly synthesized protein was analyzed by SDS/PAGE.

**Purification of Glutathione *S*-Transferase (GST)-Fused hTid-1.** The DNA fragment containing the full-length hTid-1 coding sequence amplified by PCR was subcloned into the expression plasmid pGEX-4T-1 (Amersham Pharmacia Biotech), generating pGEX-Tid1, which was expressed in *Escherichia coli* DH5$\alpha$. Overnight cultures were diluted 1:40 in 500 ml of Luria-Bertani medium with 100 $\mu$g/ml carbenicillin and incubated for 4 h at 37°C with shaking. Protein induction was achieved by the addition of 0.5 mM isopropyl-D-thiogalactopyranoside to cells in exponential phase for 4 h at 30°C. Bacterial cultures were pelleted and resuspended in PBS (140 mM NaCl/2.7 mM KCl/10 mM $Na_2HPO_4$/1.8 mM $KH_2PO_4$, pH 7.4) supplemented with 1 mM DTT and protease inhibitors (Complete; Roche). Cells were then disrupted with a French pressure cell and centrifuged to remove cell debris. The supernatants were mixed with a 50% (vol/vol) slurry of glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech) for 1 h at room temperature. The beads were washed three times with PBS; bound proteins were eluted with 10 mM reduced glutathione dissolved in 50 mM Tris·HCl (pH 8.0). The protein was >95% pure as determined by SDS/PAGE followed by staining with Coomassie brilliant blue R-250 (data not shown). Protein concentrations were determined by the Bradford method (Bio-Rad) with BSA as a standard. The recombinant GST fusion protein was used in coimmunoprecipitation experiments. The hTid-1 in which the GST tag was removed by treatment with thrombin was used for the measurement of DNA-dependent ATPase, DNA helicase, and for the electrophoretic mobility-shift assay (EMSA).

**DNA-Dependent ATPase and DNA Helicase Assays.** These assays were performed essentially as described (22) in the presence or absence of the purified hTid-1 protein. Poly(dT)$_{3000}$ served as cofactor for the DNA-dependent ATPase, and Box I with a 3′ single-stranded tail (Box I substrate) was used for the helicase assay (11).

**Coimmunoprecipitation Assay.** Coimmunoprecipitation of *in vitro* translated hTid-1 protein with the UL9 protein was carried out as follows. Defined amounts of reticulocyte lysate containing labeled hTid-1 and 150 ng of UL9 protein were mixed together in 0.5 ml of buffer A (50 mM Hepes-KOH, pH 7.8/10% (vol/vol) glycerol/1% BSA/2 mM $MgCl_2$/1 mM DTT/120 mM KCl/0.1% Nonidet P-40/0.7 $\mu$M Aprotinin/0.5 mM PMSF) and allowed to rock gently at 4°C for 1 h. After incubation, 5 $\mu$g of polyclonal anti-UL9 antibody was added to the mixture. Incubation continued with rocking for 2 h, after which 20 $\mu$l of protein A-agarose (Roche) was added and rocking continued for 2 h at 4°C. Precipitates were collected by brief centrifugation and washed 5 times with buffer B (20 mM Tris·HCl, pH 7.5/150 mM NaCl/0.1% (vol/vol) Tween 20). The pellets were resuspended in 50 $\mu$l of SDS lysis buffer (20 mM Tris·HCl, pH7.5/50 mM NaCl/0.5% SDS/1 mM DTT) and electrophoresed through an 8% denaturing polyacrylamide gel. The gel was fixed, soaked in "Amplify" (Amersham Pharmacia Biotech), and dried before being analyzed with a PhosphorImager (Molecular Dynamics). In the reciprocal experiment, 150 ng of purified GST-fused hTid-1 was incubated with increasing amounts of UL9 protein in buffer A for 1 h at 4°C. An amount equal to 150 $\mu$g of polyclonal anti-GST antibody (Rockland) was added to the mixture, and the immunocomplexes were recovered, washed, and separated by 8% SDS/PAGE as described above. After transfer to a nitrocellulose membrane (0.45-$\mu$m pore size; Schleicher & Schuell) the blot was probed with a polyclonal anti-UL9 antibody followed by incubation with an anti-rabbit IgG, coupled to horseradish peroxidase. The horseradish peroxidase signal was detected with the ECL kit (Amersham Pharmacia Biotech) and exposed to Hyperfilm.

**EMSA.** The 110-bp fragment that contained the minimal ori$_s$ sequence was excised from pCG5 (14) by digestion with the *Eco*RI and *Xba*I restriction enzymes and then labeled with the Klenow fragment of *E. coli* DNA polymerase I (United States Biochemical) and [$\alpha$-$^{32}$P]dATP [3000 Ci (1 Ci = 37 GBq)/mmol, New England Nuclear]. Reaction mixtures (20 $\mu$l) containing the indicated amounts of UL9 protein and hTid-1 in binding buffer [25 mM Hepes-KOH, pH 7.8/2.5 mM DTT/20 mM NaCl/10 $\mu$g of acetylated BSA/10% (vol/vol) glycerol/0.05% Nonidet P-40/500 ng of poly(dI-dC)·poly(dI-dC) (Amersham Pharmacia Biotech)] were preincubated at 37°C for 30 min. The labeled DNA fragment (50 fmol) was added to the mixtures. The binding reactions were conducted at 37°C for 20 min. The protein–DNA complexes were then analyzed by 5% nondenaturing polyacrylamide gel electrophoresis with TBE buffer (45 mM Tris-borate/1 mM EDTA) at 4°C. After electrophoresis, the gel was dried on Whatman DE 81 paper under vacuum and analyzed with a PhosphorImager (Molecular Dynamics).

## Results

**Identification of hTid-1 as a Binding Partner for UL9 Protein in Yeast.** To identify cellular proteins that interact with the HSV-1 UL9 protein, we screened a HeLa cell cDNA library cloned into the activation domain vector pACT2 by using the yeast two-hybrid system. To serve as the bait, we constructed a plasmid vector (pGBKUL9) that expressed a fusion protein with the DNA-binding domain of the GAL4 protein and the UL9 protein. Transformation of pGBKUL9 into the yeast strain AH109 did not activate the *lacZ* gene. About $7 \times 10^5$ colonies were screened in the two-hybrid assay, and 48 positive clones were identified. To monitor the two-hybrid interactions of positive clones, $\beta$-galactosidase activity was estimated by the colony-lift filter assay. All positive clones showed $\beta$-galactosidase activity (data not shown). Plasmids from positive clones were isolated and analyzed by DNA sequencing. Sequence analysis of 48 cDNAs revealed five encoding the p150$^{Glued}$ component of the dynactin complex, three encoding the neural F Box 42 kDa protein (NFB42), and three encoding hTid-1, a human homologue of the bacterial DnaJ protein, as well as several other genes. In the experiments to be described, we focused on the interaction of the UL9 protein with the hTid-1 because of the potential for this interaction to be part of the viral initiation process.

**Interaction of hTid-1 with UL9 Protein *in Vitro*.** The interaction between hTid-1 and the UL9 protein was confirmed by *in vitro* co-immunoprecipitation. UL9 protein was incubated with increasing amounts of *in vitro* transcribed/translated hTid-1 and immunoprecipitated with anti-UL9 antibody. The immunocomplexes were resolved by SDS/PAGE and visualized by autoradiography. As shown in Fig. 1A, the $^{35}$S-labeled hTid-1 was co-immunoprecipitated with the UL9 protein by the polyclonal UL9 protein antibody.

A similar experiment was carried out with purified GST-hTid-1 and the UL9 protein. The GST-Tid-1 was incubated with

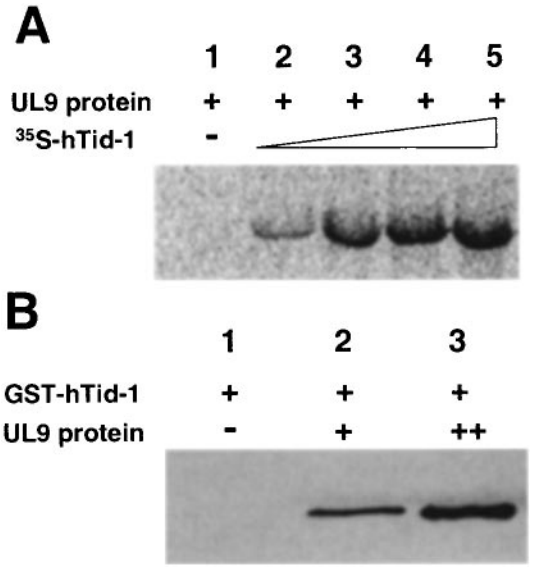


**Fig. 1.** Coimmunoprecipitation of UL9 protein with hTid-1. (*A*) hTid-1 was translated *in vitro* in the presence of [$^{35}$S]methionine. UL9 protein (150 ng) was mixed with increasing amounts of $^{35}$S-labeled hTid-1 (lane 1, 0 $\mu$l; lane 2, 10 $\mu$l; lane 3, 20 $\mu$l; lane 4, 30 $\mu$l; and lane 5, 40 $\mu$l). The mixtures were immunoprecipitated with anti-UL9 antibody, and the immunoprecipitated proteins were resolved by 8% SDS/PAGE and visualized by autoradiography. (*B*) Purified GST-hTid-1 (150 ng) was incubated with increasing amounts of UL9 protein (lane 1, 0 ng; lane 2, 300 ng; and lane 3, 600 ng). After immunoprecipitation with anti-GST antibody, the precipitates were dissolved and analyzed by 8% SDS/PAGE, transferred to a nitrocellulose membrane, and blotted with anti-UL9 antibody as described in *Materials and Methods*.

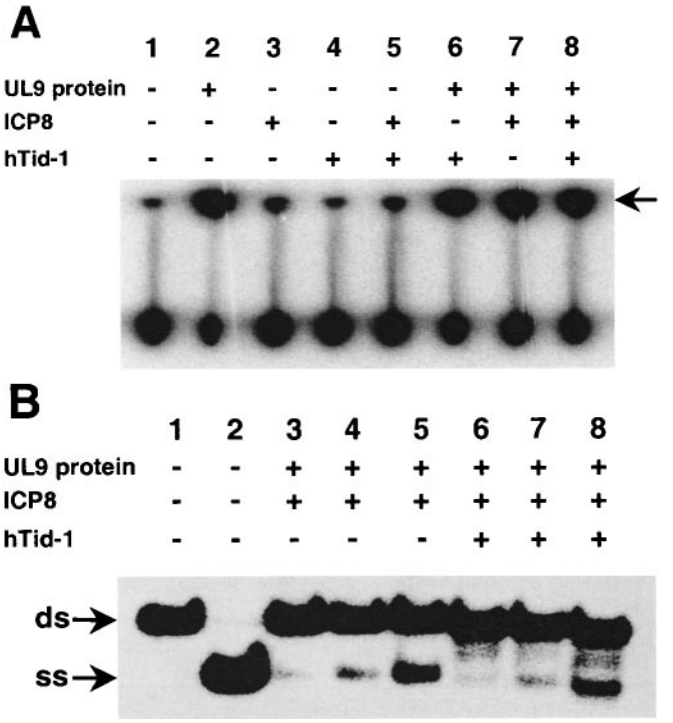


**Fig. 2.** Lack of effect of hTid-1 on DNA-dependent ATPase and helicase activities of UL9 protein. (*A*) ATPase assays were performed in a reaction mixture (25 $\mu$l) containing 4 pmol of UL9 protein, 4 pmol of ICP8, 1 $\mu$g of poly(dT)$_{3000}$, and 2 $\mu$Ci [$\gamma$-$^{32}$P]ATP, in the presence or absence of 120 ng of hTid-1 as described (22). Reaction mixtures were incubated at 37°C for 30 min and quenched by the addition of 3 $\mu$l of 0.5 M EDTA (pH 8.0). Each quenched sample (1 $\mu$l) was chromatographed on a polyethyleneimine-cellulose plate (Merck). The arrow indicates the position of free inorganic phosphate. (*B*) Helicase assays were carried out as described (22) by using the Box I DNA substrate. Incubation was without hTid-1 (lanes 3–5) or with hTid-1 (lanes 6–8) in the presence of 0.6 pmol of UL9 protein and 0.6 pmol of ICP8. Incubation was for 30 min (lanes 3 and 6), 60 min (lanes 4 and 7), and 90 min (lanes 5 and 8). Lanes 1 and 2 show non-denatured and heat-denatured Box I substrate, respectively.

increasing amounts of UL9 protein. Polyclonal anti-GST antibody was added to the mixture and the immunocomplexes were subjected to SDS/PAGE and then blotted onto a nitrocellulose membrane. When the blots were probed with a polyclonal rabbit anti-UL9 antibody, followed by incubation with an anti-rabbit IgG coupled to horseradish peroxidase, increasing amounts of UL9 protein were found to be bound to the GST-Tid-1 as increasing amounts of UL9 protein were added (Fig. 1B). Taken together, these data suggest that the interaction between hTid-1 and the UL9 protein is specific and not an artifact of the yeast two-hybrid system.

**Lack of Effect of hTid-1 on DNA-Dependent ATPase and DNA Helicase Activities of UL9 Protein.** The UL9 protein possesses DNA-dependent ATPase and 3′ to 5′helicase activities, in addition to its origin binding activity (6, 10). To determine whether hTid-1 affects these activities, DNA-dependent ATPase, and DNA helicase assays (11) were carried out in the presence or absence of hTid-1. hTid-1 had no effect on either the DNA-dependent ATPase or the Box I specific helicase activity of the UL9 protein (Fig. 2).

**Effect of hTid-1 on Binding of UL9 Protein to the ori$_s$.** The effect of hTid-1 on the origin binding activity of UL9 protein was determined by EMSA (Fig. 3A). A labeled 110-bp ori$_s$-containing DNA fragment was mixed with UL9 protein that had been preincubated in the presence (Fig. 3A, lanes 5–8) or absence (Fig. 3A, lanes 2–4) of hTid-1. Addition of increasing amounts of the UL9 protein resulted in the increased formation of dimeric and multimeric UL9 protein-ori$_s$ complexes (Fig. 3A, lanes 2–4). The labeled complexes disappeared on addition of an excess of unlabeled 110-bp ori$_s$-containing DNA fragment, indicating that the binding was sequence-specific (data not shown). The dimeric UL9 protein-ori$_s$ complex predominated and the amount formed was directly proportional to the UL9 added (Fig. 3B). When the UL9 protein was preincubated with increasing amounts of hTid-1, and the ori$_s$-containing DNA fragment added, the hTid-1 significantly enhanced the binding of UL9 protein to ori$_s$ (compare Fig. 3A, lane 2 to lanes 5–8). However, in contrast to the reaction in which UL9 protein alone was added, there was a marked enhancement of formation of the multimeric UL9 protein-ori$_s$ complex, and a corresponding decrease in formation of the dimeric UL9 protein-ori$_s$ complex (Fig. 3A and 3C). hTid-1 did not bind to the ori$_s$-containing fragment (Fig. 3A, lane 9). Formation of multimeric complex appeared to be cooperative. As shown in Fig. 3A, migration of the complexes was not altered in the presence of hTid-1, indicating that hTid-1 was not retained in UL9 protein-ori$_s$ complexes.

## Discussion

The UL9 protein encoded by HSV-1 is essential for viral DNA replication and very likely functions as a replication initiator at the origins of DNA replication (1, 2). The UL9 protein shares a number of properties with the SV40 Tag and the HPV E1 protein, including origin-specific binding, DNA-dependent ATPase and helicase activities (7–9). Like the Tag and the E1 protein, the UL9 protein binds to the cellular DNA polymerase-$\alpha$ primase (21, 23, 24). During SV40 DNA replication, the interaction between Tag, the single strand DNA binding protein (RP-A), topoisomerase I, and

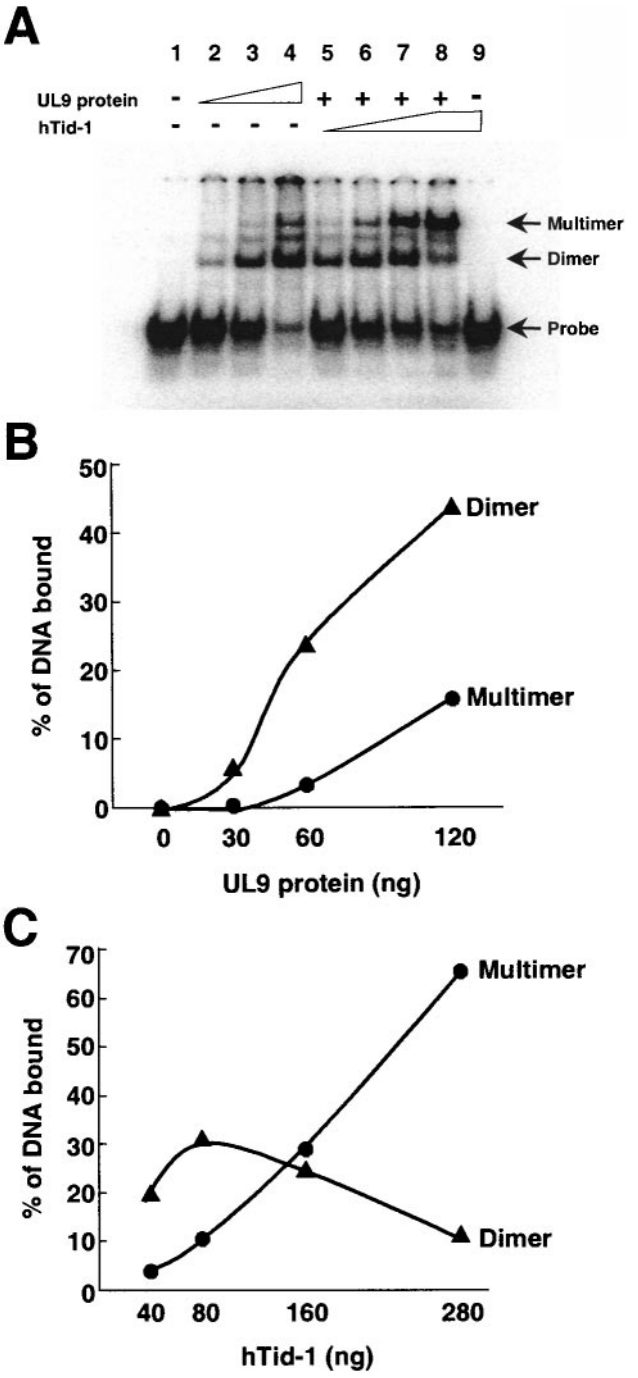


**Fig. 3.** Effect of hTid-1 on the binding of UL9 protein to $ori_s$. (*A*) EMSA was performed with 50 fmol of $^{32}$P-labeled 110-bp DNA fragment containing the minimal $ori_s$ sequence. The labeled probe was incubated in the absence of hTid-1 with increasing amounts of UL9 protein (lane 2, 30 ng; lane 3, 60 ng; and lane 4, 120 ng) or with increasing amounts of hTid-1 (lane 5, 40 ng; lane 6, 80 ng; lane 7, 160 ng; and lanes 8 and 9, 280 ng) in the presence of 30 ng of UL9 protein. The reaction products were resolved by non-denaturing gel electrophoresis as described in *Materials and Methods*. The appearance of dimers (▲) and multimers (●) in the absence of hTid-1 (*B*) and in the presence of hTid-1 (*C*) was quantified with a PhosphorImager (Molecular Dynamics).

DNA polymerase α-primase are essential for the formation of the replication initiation complex (25–28). To date, numerous attempts to reconstitute origin-dependent HSV-1 DNA replication *in vitro* with the seven purified viral proteins and cellular proteins such as DNA ligase, topoisomerase, and DNA polymerase α-primase have been unsuccessful, suggesting that other cellular proteins are required (1, 2).

We have used the yeast two-hybrid system to screen a HeLa cell cDNA library to identify cellular proteins that interact with the HSV-1 UL9 protein. Proteins identified in this screen included the $p150^{Glued}$ component of the dynactin complex (18), a protein that is highly expressed in adult neural tissue, and functions in mitosis, and NFB42 also highly enriched in the nervous system and postulated to play a role in maintaining neurons in a postmitotic state (19). Because of their location, the interaction of these proteins with the UL9 protein may be related to the latency of HSV-1 in neural tissue (1, 29). The other protein identified in the screen, hTid-1, is a human homologue of Tid56 encoded by the *Drosophila melanogaster* tumor suppressor gene *l* (2)*tid* (20). In *Drosophila*, imaginal disk null mutants of Tid56 fail to differentiate, resulting in the development of lethal tumors (30, 31). The human hTid-1 was initially cloned during the screening of a HeLa cDNA library to identify proteins that interact with the HPV type 16 E7 oncoprotein (20). hTid-1 belongs to the type I DnaJ chaperone family of proteins (32), with three conserved domains, a N-terminal J domain, a glycine/phenylalanine-rich region, and a cysteine-rich region containing CXXCXGXG motif.

Although, hTid-1 had no effect on the DNA-dependent ATPase and helicase activities of UL9 protein, it enhanced the binding of the UL9 protein to $ori_s$, and facilitated the cooperative formation of the multimer from the dimeric UL9 protein-$ori_s$ complex. The SV40 Tag forms a dihexamer that surrounds the DNA at the SV40 origin (33, 34). HPV E1 protein binds to DNA mainly as a hexamer (35). However, in the presence of Hsp40 (a homologue of bacterial DnaJ protein), the E1 protein also bound to DNA as a dihexamer. One possible role for hTid-1 that is supported by the EMSA is to facilitate formation of the analogous higher order complexes of UL9 protein. These multimeric, possibly di-dimeric forms may represent the replication-competent form of the UL9 protein during initiation of HSV-1 DNA replication.

A report has been recently appeared showing the stimulation of the binding of the UL9 protein to the HSV-1 $ori_s$ by the human and *E. coli* DnaJ proteins (36).

There are several mechanisms by which hTid-1 can enhance the binding of UL9 protein to $ori_s$. The first possibility is that the hTid-1 functions as a chaperone to refold misfolded UL9 proteins in the baculovirus expressed UL9 protein preparation. This possibility is unlikely, because incubation of the UL9 protein with hTid-1 did not enhance the DNA-dependent ATPase or helicase activities. A second possibility is that hTid-1 is an integral part of an origin recognition complex. This possibility is also unlikely, because hTid-1 did not cause the formation of new, more slowly migrating species in the EMSA analysis. The third possibility is that the hTid-1 chaperone may somehow induce a conformational change in the UL9 protein and thereby facilitate the formation of a multimeric UL9 protein-$ori_s$ complex from the dimeric UL9 protein. This role for hTid-1 is analogous to the chaperone function proposed for the assembly of the replication initiation complex in *E. coli* and bacteriophage lambda (37–39).

In contrast to SV40, polyoma virus and HPV, HSV-1 encodes most of the enzymes that are required for the replication of its genome. Nevertheless, our finding that the HSV-1 UL9 protein reacts specifically with a cellular chaperone to enhance binding to its target DNA, taken together with the previous report that the UL9 protein binds the cellular DNA polymerase α-primase (18), suggests that cellular proteins may play a more important role in viral DNA replication than had previously been recognized.

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