Herpes simplex virus type 2 UL14 gene product has heat shock protein (HSP)-like functions

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Summary

The HSV-2 UL14 gene encodes a 32 kDa protein that is a minor component of the viral tegument. The protein relocates other viral proteins such as VP26 and UL33 protein into the nuclei of transiently coexpressing cells (Yamauchi et al., 2001). We found that the protein shared some characteristics of heat shock proteins (HSPs) or molecular chaperones, such as nuclear translocation upon heat shock, ATP deprivation and osmotic shock. Interestingly, a significant homology over a stretch of 15 amino acids was found between an N-terminal region of HSV UL14 protein and the substrate-binding domain of Hsp70 family proteins. Two arginine residues in this region were important for nuclear translocation of VP26. In addition, overexpression of UL14 protein increased the activity of coexpressed firefly luciferase, which suggested that the protein functioned in the folding of newly synthesized luciferase. We thus conclude that UL14 protein can act as a chaperone-like protein in a singly expressed state.

Key words: HSV-2 UL14, Heat shock, Molecular chaperone, Hsp70, Luciferase activity

Introduction

A molecular chaperone is 'a protein that binds to and stabilizes an otherwise unstable conformer of another protein and, by controlled binding and release of the substrate protein, fascilitates its correct fate in vivo: be it folding, oligomeric assembly, transport to a particular subcellular compartment, or controlled switching between active/inactive conformations' (Hendrick and Hartl, 1993). Many kinds of environmental stress, such as heavy metals, ethanol, amino acids analogues, anoxia and agents capable of pertubing protein structure, cause a similar response (Ohtsuka and Hata, 2000). Molecular chaperones can temporarily stabilize unfolded or partially folded proteins, which reduces the free concentration of aggregation-sensitive folding intermediates and effectively prevents aggregation processes both in vivo and in vitro (Jakob and Buchner, 1994).

The ubiquitous and abundant Hsp70 chaperones, with their co-chaperones, have been proposed to be required for the co-translational folding of cytosolic proteins (Langer et al., 1992; Hartl, 1996; Mayhew and Hartl, 1996). Hsp70 proteins rely on their ability to associate with short hydrophobic segments of unfolded substrate polypeptides in an ATP-controlled fashion (Rüdiger et al., 1997; Bukau and Horwich, 1998). The best in vitro evidence for a role of Hsp70 in folding of newly synthesized polypeptides has been obtained for firefly luciferase translated in reticulocyte lysates that are depleted of Hsp70 or its DnaJ co-chaperone, Hsp40 (Frydman et al., 1994). Depletion results in a specific enzymatic activity of translated luciferase that is decreased by 70%. Restoration of full enzymatic activity is possible only when Hsp70 and Hsp40 are re-added before onset of translation, indicating a co-translational mode of action of Hsp70 and Hsp40 in the folding of firefly luciferase in this cell-free system (Frydman et al., 1994). A different class of chaperones, the TriC and GroEL chaperonins, is known to assist the de novo folding of cytosolic proteins (Horwich et al., 1993; Frydman and Hartl, 1996; Lewis et al., 1996; Ewalt et al., 1997; Farr et al., 1997). For GroEL of *E. coli*, a post-translational role in folding of a subset of 5-15% of newly synthesized proteins has been shown (Bochkareva et al., 1988; Horwich et al., 1993; Gaitanaris et al., 1994; Reid and Flynn, 1996; Ewalt et al., 1997). There are implications that Hsp70 chaperones and the chaperonins provide a protected folding environment for nascent chains (Frydman and Hartl, 1996).

HSPs, particularly those of the Hsp70 family, are transcriptionally induced by and associate with viral proteins. Infections with adenovirus (Wu et al., 1986), vaccinia virus (Sedger and Ruby, 1994), cytomegalovirus (Colberg-Poley and Santomenna, 1988), simian virus 40 (SV40) and polyomavirus (Khandijan and Türler, 1983) induce mRNAs for Hsp70 family proteins. Because of the diverse functions of Hsp70-like proteins and their increased expression and association with viral proteins during viral infections, it is likely that Hsp70 assists in aspects of virion assembly as a cellular chaperone protein (Cripe et al., 1995). An HSV-encoded true late gene product, US11, has been shown to enhance survival of cells to heat shock when it is overexpressed, although heat treatment did not modify the intracellular distribution of the protein (Diaz-Latoud et al., 1997). It seems practical for the virus to encode its own HSP or molecular chaperone.

The herpes simplex virus (HSV) genome contains at least 74 different known protein-coding genes (Dolan et al., 1998; McGeoch et al., 1988). The UL14 gene of HSV type 1 (HSV-1) and type 2 (HSV-2) encodes a 32 kDa protein expressed late in infection that is a minor component of the virion tegument (Wada et al., 1999; Cunningham et al., 2000), and the coding region overlaps that of UL13, which encodes a protein kinase (Dolan et al., 1998; Daikoku et al., 1997). Most conserved residues are located in the nonoverlapping region, and the overlapping region seems to encode a variable length C-terminal domain that is poorly conserved in sequence. A UL14-deficient mutant exhibits an extended growth cycle at low multiplicities of infection and appears to be compromised in efficient transit of virus particles from the infected cell. In mice injected intracranially, the 50% lethal dose of the mutant was reduced more than 30,000-fold, suggesting that the protein contributes to successful viral growth and thus to the sophistication of the herpesvirus life cycle (Cunningham et al., 2000). The versatile localization of UL14 protein in singly expressed cells and the ability to relocate viral proteins into the nucleus by coexpression may be only a part of its intrinsic functions (Yamauchi et al., 2001). In this study we suggest that HSV-2 UL14 protein has HSP-like functions.

Materials and Methods

Cells

African green monkey kidney Vero cells and human HEp-2 cells were propagated in Dulbecco's modified Eagle's medium containing 8% fetal calf serum (FCS). Cell lines constitutively expressing HSV-2 UL14 protein were constructed in Vero and HEp-2 cells as follows. Exponentially growing cells in 35 mm plates were transfected with pcDNA3-UL14 and maintained at 37°C. After 48 hours, cells were trypsinized and passed onto 60 mm plates and propagated in medium containing 1 mg/ml of G418 (Sigma). Colonies were isolated by attaching cells to small-cut, sterilized filtering paper soaked in trypsin. The expression of UL14 protein was observed by indirect immunofluorescence and western blotting.

Plasmids

PCR primers (forward, F; reverse, R) designed to construct plasmids expressing deletion mutants of the UL14 protein with incorporated restriction enzyme sites were as follows. PcDNA3-UL14ND20 (F, GAGAAAGCTTTCATGGCCGAGGTGTAC, *Hin*dIII); pcDNA3-UL14ND60 (F, GAATAAGCTTCGATGCTAAAGTCCC, *Hin*dIII); pcDNA3-UL14ND120 (F, CAGGAAGCTTAGATGGAAGAGGC-CG, *Hin*dIII). The reverse primers for these plasmids were: C14R, GACGGGATCCTCACTCGCCATCGGG, *Bam*HI; PcDNA3-UL14 D(51-90) (F, CGACGTGATATCCTAACCGCACACCGACGGTAC-CT, *Eco*RV; R, GTGGGCGATATCGGCGGCCATAAAGGCGCCA, *Eco*RV); pcDNA3-UL14D(121-180) (F, CGCGGCGATATCCCG-GACGCCCAAGCGGCCC, *Eco*RV); R, GCCGCCGATATCGAGC-TGCTCCTCCTGGTC, *Eco*RV). Point mutations were incorporated according to the instructions of QuikChange Site-Directed Mutagenesis Kit (Stratagene). The primers constructed and the double stranded DNA templates used were as follows. PcDNA3-UL14K51A (F, GCCTTTATGGCCGCCGCGGCGGCCCACTTGGAAT; R, TTCCAAGTGGGCCGCCGCGGCGGCCATAAAGGC; template, pcDNA3-UL14); pcDNA3-UL14R60A (F, GGAATTGGAGGCGG-CGCTAAAGTCCCGCGCGCGCTTAG; R, CTAAGCGCGCGCGG-GACTTTAGCGCCGCCTCCAATTCC; template, pcDNA3-UL14); pcDNA3-UL14R(60,64)A (F, GCGGCGCTAAAGTCCGCCGCGC-GCTTAGAGATG; R, CATCTCTAAGCGCGCGGCGGACTTAGC-GCCGC; template, pcDNA3-UL14R60A); pcDNA3-UL14PM3 was constructed with primers UL14K51F, R using pcDNA3-UL14R(60,64)A as template. The nucleotide sequence of each product was verified by using the sequence analyzer ABI PRISM 310 Genetic Analyzer (PE Biosystems). Other plasmids mentioned in this study have been noted elsewhere (Wada et al., 1999; Yamauchi et al., 2001). The luciferase-expressing plasmid pGL3-Promoter vector (referred to as pGL3-p in the text) was obtained from Promega.

Antibodies

Anti-UL14 polyclonal Ab and mAb, and the anti-FLAG mAb have been described previously (Wada et al., 1999; Yamauchi et al., 2001). Anti-Hsp70 rabbit polyclonal Ab was kindly provided by K. Ohtsuka (Chubu University, Kasugai, Japan). Mouse anti-Hsp70/Hsc70 mAb was purchased from MBL. Anti-nucleolin mAb was purchased from Transduction Laboratories, anti-luciferase rabbit polyclonal Ab from Rockland, anti-PML mAb from MBL and anti-NuMA mAb from Oncogene. The secondary antibodies used were fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG and tetramethylrhodamine isothiocyanate (TRITC)-conjugated mouse anti-rabbit IgG (Sigma).

Chemicals, heat shock, ATP depletion and hyperosmotic shock assays

MG132 was obtained from Sigma. All cells were grown to a monolayer on glass coverslips in 35 mm dishes. For heat-shock assays, the dishes were sealed with parafilm and heat shocked in a water bath heated to appropriate temperatures. For recovery from heat shock, the dishes were removed from the bath and incubated at 37°C for appropriate times. Leptomycin B was used at a final concentration of 10 ng/ml. For ATP depletion assays, the medium was replaced with an ATP depletion cocktail consisting of 10 mM of sodium azide and 10 mM of 2-deoxy-D-glucose and incubated at 37°C for 5-10 hours. In order to induce hyperosmotic shock, the medium was supplemented with sorbitol to a final dilution of 1 M and incubated at 37°C for 5 hours. For fixation, the glass coverslips were washed thoroughly in PBS and fixed in cold acetone for 5 minutes. The cells were washed extensively in PBS and left to dry and stored in a –80°C freezer.

Immunofluorescence and immunoblotting

For indirect immunofluorescence, the primary antibodies were diluted in PBS. Antibodies were used at dilutions as follows: anti-UL14 polyclonal Ab, 1/100-1/300; anti-UL14 mAb, 1/100; anti-Hsp70 rabbit polyclonal Ab, 1/80; anti-Hsp70/Hsc70 mouse mAb, 1/10; anti-FLAG mAb, 1/100; anti-nucleolin mAb, 1/100; anti-PML mAb, 1/100; and anti-NuMA mAb, 1/100. After incubation at 37°C for 30 minutes, the coverslips were washed extensively in PBS then treated with secondary antibodies that were all diluted at 1/150. After a further 30 minute incubation, the coverslips were washed in PBS and mounted using Perma Fluor (Immunon). Where appropriate, DNA was stained (after RNAase treatment) using propidium iodide at a concentration of 1 μg/ml for 5 minutes after the secondary antibody incubations. Samples were examined with Zeiss laser scanning microscope LSM510 or with the Bio-Rad MRC series confocal imaging system. As primary antibodies for immunoblotting, anti-Hsp70 rabbit polyclonal Ab was used at 1/5000, and anti-UL14 polyclonal Ab at 1/2000.

Antibody microinjection

Cells were grown to a monolayer on glass coverslips in 35 mm dishes. The antibody was microinjected through a glass capillary into the cytoplasm of cells. After incubation at 37°C for 2 hours, cells were heat shocked and fixed as described, and detected by immunofluorescence.

Transfection of oligomers

Oligomers were obtained from Sigma genosys. Hsp70 antisense oligomer (5′-CGCGGCTTTG-GCCAT-3′) was complementary to the initiation codon and 4 downstream codons of human Hsp70 mRNA. The corresponding sense oligomer (5′-ATGGCCAAAGCCGCG-3′) and nonsense oligomer (5′-CGGGTATGCTTCGCC-3′) were used as controls (Wei et al., 1995). The oligomers were diluted to 200 μM and stored at –20°C. For cotransfection assays, oligomers were added to the transfection mixture to a final concentration of 0.5 μM (Gibco BRL).

Plasmid transfection and luciferase assays

For plasmid transfection, monolayers of cells seeded 24 hours earlier were transfected with the Lipofectamine reagent (Gibco-BRL) according to the instructions of the supplier. For luciferase assays, the Luciferase assay system (Promega) was used. 24 hours after transfection of pGL3-promoter containing mixtures, 35 mm dish cells were washed twice in PBS, harvested as recommended by the supplier. Luciferase activity was measured for 10 seconds with a TD-20/20 Luminometer (Turner Designs) with a 100:20 μl ratio mixture of luciferase assay reagent and cell culture lysate.

Results

The dynamic change of the localization of HSV-2 UL14 protein in response to stress

UL14 protein can relocate other viral proteins such as VP26 (the minor capsid protein encoded by the UL35 gene) and UL33 (cleavage and packaging) protein into the nuclei of coexpressing cells (Yamauchi et al., 2001). It seemed unnatural for one viral protein to interact with several other viral proteins just to translocate them into nuclei; therefore, we hypothesized that UL14 protein possessed a molecular chaperone-like function. We investigated this hypothesis by focusing on the intracellular localization of UL14 protein after stress stimulation such as heat shock.

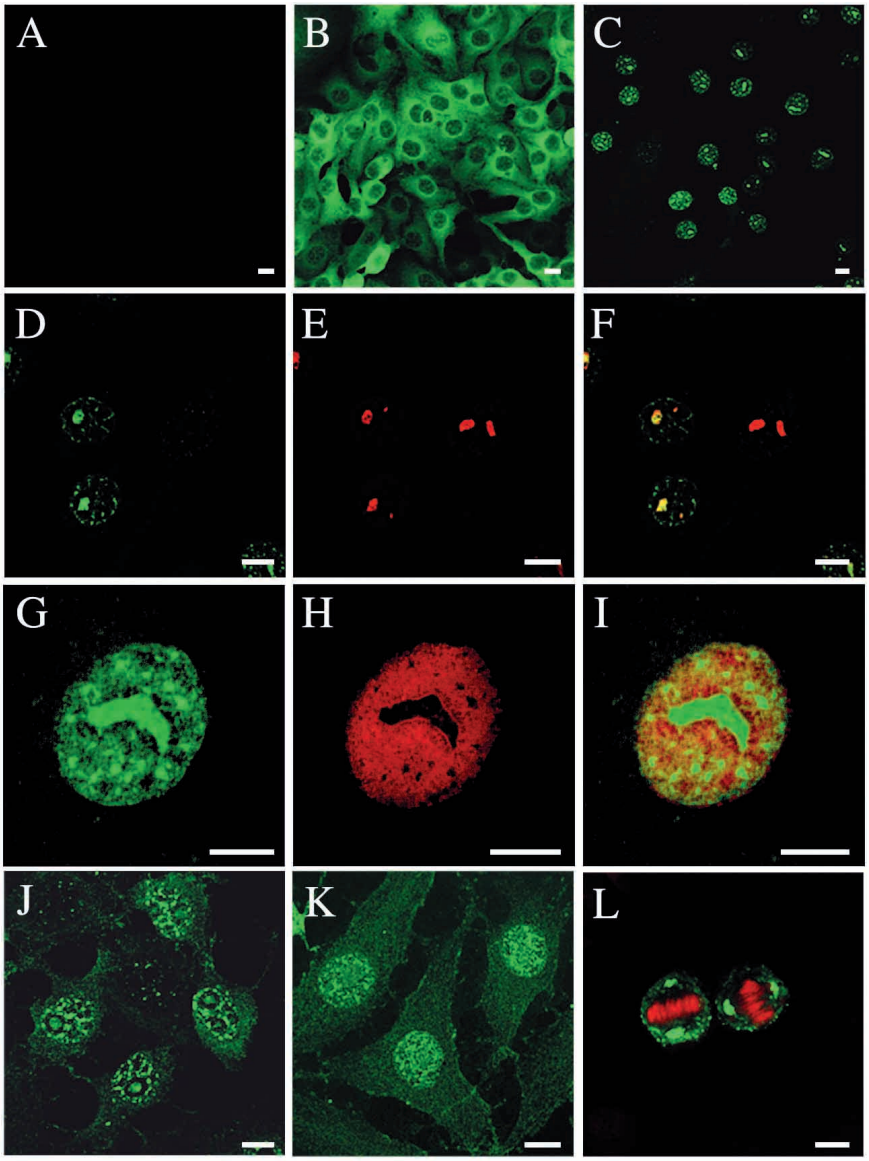

Fig. 1. The intracellular localization of UL14 protein in 14/Vero cells (A-K) and 14/HEp-2 cells (L) detected by immunofluorescence (A, mock). UL14 protein at 37°C (B) and after heat shock at 43°C for 30 minutes (C). UL14 protein (D) colocalizes with nucleolin (E) after heat shock (F, merge). UL14 protein (G) co-stained with propidium iodide (H) after heat shock (I, merge). UL14 protein localization after 5 hours treatment with ATP-depletion cocktail (J) and 5 hours of 1 M sorbitol treatment (K). UL14 protein localization in M-phase cells after heat shock, co-stained with propidium iodide (L, merged image) shows localization at the centrosomes. Bars, 10 μm.

We constructed Vero and HEp-2 cell lines that constitutively expressed UL14 protein (14/Vero and 14/Hep-2) and detected the localization of UL14 protein by indirect immunofluorescence. Cells propagated at 37°C showed mainly cytoplasmic staining (Fig. 1B). We next treated the cells with 43°C heat shock for 30 minutes. Much to our surprise, UL14 protein showed predominant nuclear staining, which was observed with more contrast in 14/Vero cells (Fig. 1C). If UL14 was a shuttling protein that continuously moves between the nucleus and cytoplasm at 37°C, it could be that its nuclear accumulation by heat shock was due to the block of nuclear export. To rule out this possibility, 14HEp-2 cells were treated with the export inhibitor leptomycin B. UL14 protein still showed mainly cytoplasmic localization showing that nuclear accumulation was due to its import into the nucleus (data not shown). UL14 protein also colocalized with nucleolin in a double-staining experiment confirming its nucleolar accumulation (Fig. 1D-F). In addition, cells were double-stained for UL14 protein and DNA using propidium iodide after RNAase treatment. We found that UL14 protein was mostly distributed in nuclear space excluding DNA, in general, the nucleolus and the nuclear matrix (Fig. 1G-I).

Heating cells that were transfected with the UL14 gene (data not shown), or treating the cell lines with an ATP-depletion cocktail (consisting of sodium azide and 2-deoxy-D-glucose)

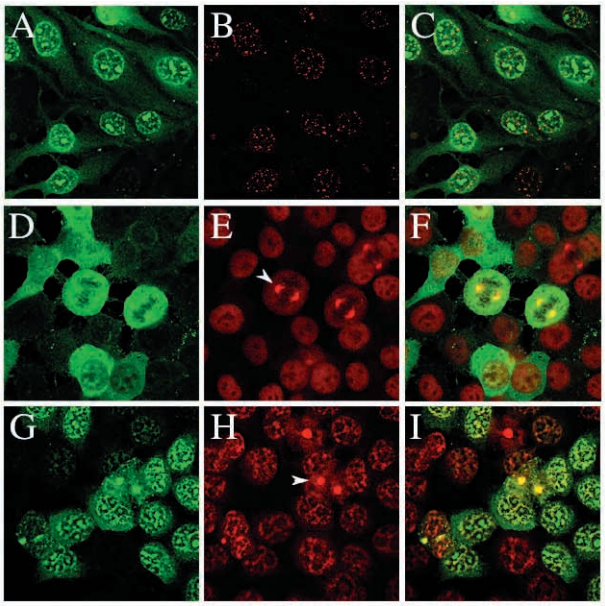

Fig. 2. UL14 protein colocalizes with cellular protein at 37°C (D-F) and after heat shock (A-C,G-I). 14/Vero cells were detected for UL14 protein (A) and PML (B) after heat shock (C, merge). 14/HEp-2 cells were detected for UL14 protein (D,G) and NuMA (E,H) at 37°C (D-F), and after heat shock (G-I); F and I are the merged images. Arrowheads indicate centrosomal localization of NuMA in M-phase cells.

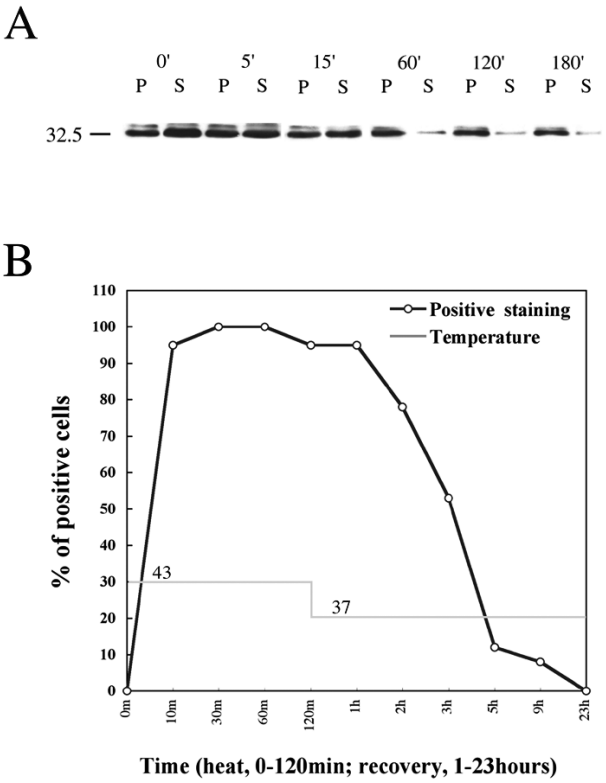

Fig. 3. (A) Fractionation of 14/HEp-2 cells during continuous heat shock at 43°C. Cells were heat-shocked for up to 180 minutes, and fractionated into insoluble (pellet, P) and soluble (supernatant, S) fractions at 0, 5, 15, 60, 120 and 180 minutes with PBS containing 1% Triton X-100. The samples were separated by SDS-PAGE, subjected to western blotting and finally detected with anti-UL14 polyclonal antibody. The total amount of the protein decreased after 60 minutes of heat shock, but the amount of UL14 protein in the insoluble fractions was sustained. (B) The nucleolar localization of UL14 protein in 14/Vero cells during heat shock at 43°C and recovery at 37°C. Cells were heat-shocked at 43°C for 120 minutes and left to recover at 37°C for up to 23 hours. Cells were fixed at several time points for immunofluorecence. Bright nucleolar staining of UL14 protein was counted and the population was plotted against time. Nearly all of the cells exhibited nucleolar localization of UL14 protein after 10 minutes of heat shock. The protein gradually delocalized from the nucleolus and, subsequently, from the nucleus during recovery at 37°C.

also exhibited nuclear relocation of UL14 protein (Fig. 1J). Furthermore, hyperosmotic stress induced by 1 M sorbitol treatment retrieved similar, but non-identical, results (Fig. 1K). Strikingly, in M phase 14/Hep-2 cells, we found that UL14 protein localized markedly at centrosomes (Fig. 1L). In heat-shocked 14/Vero cells, UL14 protein colocalized with PML (Fig. 2A-C). In 14/Hep-2 cells, UL14 protein colocalized with the nuclear mitotic apparatus protein (NuMA) at 37°C (Fig. 2D-F). In M-phase cells after heat shock (Fig. 2G-I), the colocalization became extremely clear, suggesting that the UL14 protein associates with NuMA or centrosome after heat shock. PML colocalization was not seen in 14/Hep-2 cells after heat shock, but was observed after 5 hours of treatment with 20 μM of proteasome-inhibitor MG132 (data not shown).

These rapid changes in intracellular localization of UL14 protein were intriguing. We next analyzed the solubility of the protein when undergoing continuous heat stress. Exponentially growing 14/HEp-2 cells were heated to and maintained at 43°C for 3 hours. Cells were lysed in PBS containing 1% Triton X-100 and fractionated into soluble and insoluble fractions at appropriate time points and subjected to western blotting (Fig. 3A). The ratio of the insoluble fraction of UL14 protein increased greatly after 60 minutes of heat treatment. Also, the overall amount of the protein decreased in cells heated for a longer time.

Localization of UL14 protein and Hsp70 in heat-shocked 14/Vero and 14/Hep-2 cells

It is known that cellular HSPs such as Hsp70 return to the cytoplasm during a recovery period at 37°C after heat shock at 43°C (Hattori et al., 1993). We examined whether this was the case for UL14 protein localization. When the temperature was maintained at 37°C after heat shock, UL14 protein began to delocalize to the cytoplasm (data not shown). This phenomenon pointed out that the localization of UL14 protein indeed was similar to that of Hsp70. To understand the movement of UL14 protein, cells were heated at 43°C for 2 hours and recovered at 37°C for up to 23 hours. Cells were fixed at certain time points, detected by immunofluorecence for UL14 protein, and brightly stained nucleoli were counted as ‘positively-stained cells’. The percentage of positive cells was plotted against time during and after heating. Fig. 3B shows that nucleoli-positive cells decreased during the recovery period and dropped to 10% at 5 hours.

Cells double-stained with UL14 protein and Hsp70 after heat

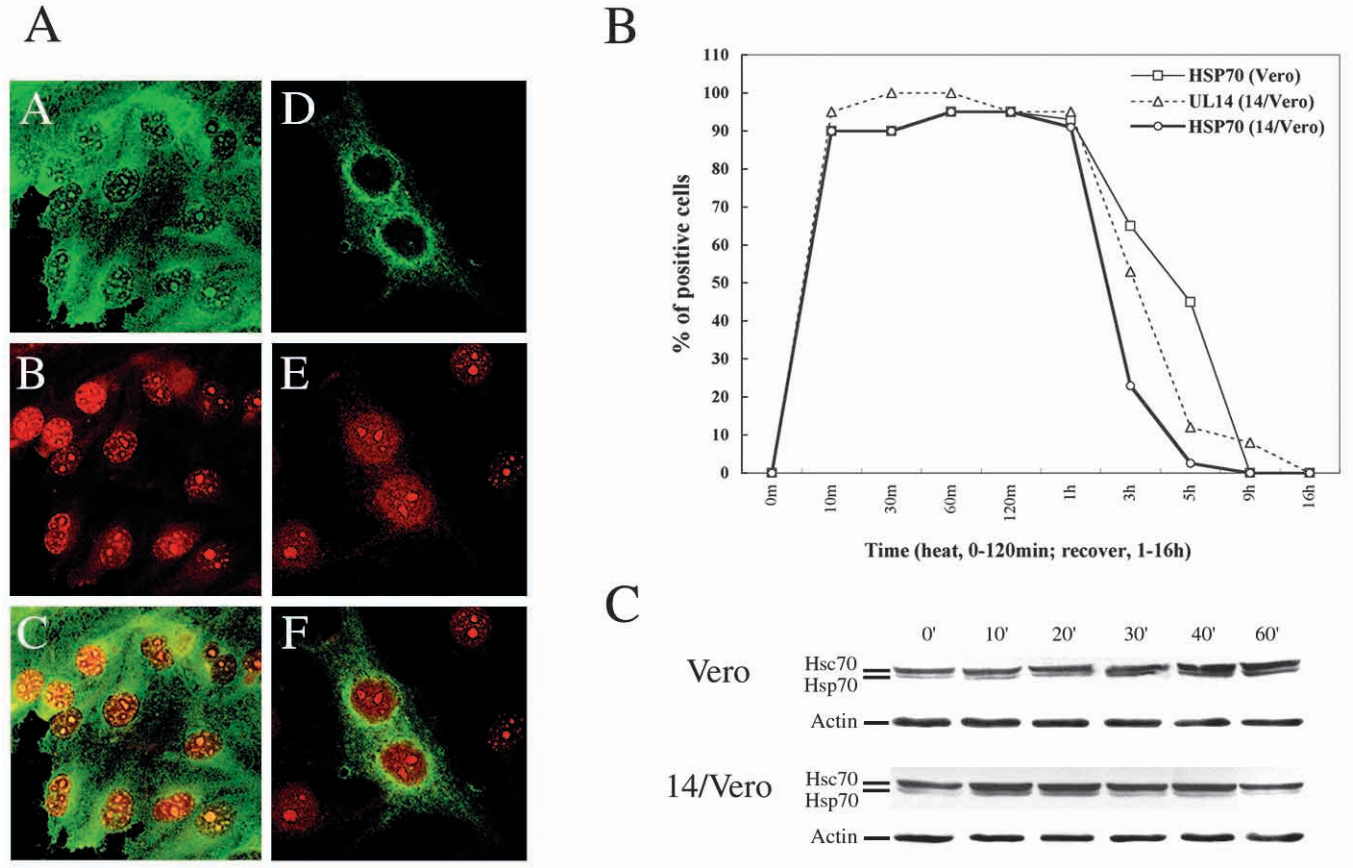

Fig. 4. (A) Immunofluorescence of 14/Vero cells after heat shock. (A-C) Heat-shocked cells were detected for Hsp70 (A) and UL14 protein (B), showing that the two proteins colocalize in the nucleus and nucleolus (C, merge). (D-F) 14/Vero cells were microinjected with anti-Hsp70 polyclonal antibody and treated with heat shock. The cells were detected for the injected antibody (D) and UL14 protein (E; F, merge). UL14 protein still translocated into the nucleus and nucleolus in microinjected cells, indicating that its localization did not depend on Hsp70 function. (B) The comparison of nucleolar localization of UL14 protein and Hsp70 in Vero and 14/Vero cells. Vero and 14/Vero cells were heat-shocked at 43°C for 120 minutes and recovered at 37°C for up to 16 hours. Cells were fixed at several time points for immunofluorecence. Bright nucleolar staining of Hsp70 and/or UL14 protein was counted and the population was plotted against time. In both cells, Hsp70 rapidly translocated to the nucleoli after heat shock and started to delocalize to the cytoplasm during recovery. The rate of delocalization was faster in 14/Vero cells than in Vero cells. In 14/Vero cells, UL14 protein remained in the nucleoli for a longer time than Hsp70. (C) Hsc70/Hsp70 expression in heat-shocked Vero and 14/Vero cells. Cells were continuously heat-shocked at 43°C for up to 60 minutes and samples were collected for immunoblotting at the intervals shown. Anti-Hsp70 Ab was used at a dilution of 1/5000. To show that nearly equal amounts of protein were loaded, bands corresponding to actin were obtained from staining identical SDS-PAGE gels with CBB.

shock showed that the proteins colocalized completely in the nucleus (Fig. 4A, A-C). To see if UL14 protein associated with Hsp70 for its transport, cells were microinjected with anti-Hsp70 polyclonal antibody prior to heat shock. After heat shock at 43°C for 30 minutes, the cells were fixed and detected for UL14 protein and the injected antibody. UL14 protein translocated to the nuclei and nucleoli in microinjected cells, indicating that this process did not depend on the function of Hsp70 (Fig. 4A, D-F). Staining these cells with an anti-Hsp70 monoclonal antibody showed that injection of the polyclonal antibody inhibited Hsp70 from entering the nucleolus after heat shock (data not shown), indicating that antibody-injection knocked out Hsp70 function. To compare the dynamics of UL14 protein and Hsp70 nucleolar localization upon heat shock, Vero and 14/Vero cells were heat-shocked at 43°C for 2 hours and allowed to recover at 37°C for up to 16 hours. Samples were fixed at several time points and detected for Hsp70 and UL14 protein nucleolar localization. The plots of the experiment show that positive nucleolar staining of UL14 protein and Hsp70 was observed almost simultaneously after a short period of heating in 14/Vero cells (Fig. 4B). During the recovery period at 37°C positive staining of Hsp70 decreased more rapidly in 14/Vero cells than in Vero cells. In comparison with 14/Vero cells, a time lapse of about 4 hours was observed in Vero cells before Hsp70 delocalized completely to the cytoplasm in all cells. The localization of UL14 protein in 14/Vero cells was similar to that of Hsp70 but delocalization from the nucleoli took more time than Hsp70. In addition, nucleolar staining of UL14 protein was much brighter than that of Hsp70 in early times of heat shock (data not shown). Cells were submitted to continuous heat shock at 43°C and assayed for Hsc70/Hsp70 protein by western blotting at 0, 10, 20, 30, 40, and 60 minutes (Fig. 4C). Hsp70 gradually increased over time in Vero cells whereas Hsp70 in 14/Vero cells decreased after 30 minutes of heat shock.

Delocalization of Hsp70 from the nucleus after heat shock

(presumably upon completion of recovery) is associated with cell survival while failure to delocalize is associated with cell death (Roti Roti et al., 1998). In addition, in heat-resistant and thermotolerant cells Hsp70 delocalization from the nucleus occurs faster, while in heat-sensitive cells delocalization occurrs slower than in cells of normal heat resistance (Laszlo et al., 1993). Our result suggests that the expression of UL14 protein may contribute to cellular recovery from heat shock.

There is a homology between the substrate-binding domain of Hsp70 and an N-terminal region of HSV UL14

In the course of this research, we were curious whether UL14 had inherited a heat-shock-protein-like sequence from the natural host and so we searched for homologous amino acid sequences between UL14 and several heat shock proteins such as the small HSPs, Hsp60, Hsp70 and Hsp90. Interestingly, HSV UL14 protein possessed an amino acid sequence that was homologous to a part of the peptide-binding domain conserved in the Hsp70 family (Fig. 5A). The homologous domain of HSV-2 UL14 protein is a stretch of 39 amino acids starting from the Ala49. In particular, 9 out of 15 amino acids [(60) R-L-X-S-R-X(2)-L-E-X-M-X-Q-X-A (74)] in a stretch beginning at the Arg60 of HSV-2 UL14 protein shared homologies with the α-helical domain of Hsp70 located at the C-terminal end of the substrate recognition domain. These sequences were conserved only in HSV-1 and -2 among α-herpesviruses, indicating the possibility that only HSV inherited the sequences. It also seemed possible that the amino acids were important for the HSP-like properties of UL14 protein. We decided to look into these amino acids for their association with the function of UL14 protein.

Table 1. Plasmids used in the study for expression of UL14 mutant proteins

Plasmid (pcDNA3-)	UL14 mutant protein (amino acids)
UL14	Full-length HSV-2 UL14 (1-219)
UL14ND20	N-terminus shortened UL14 (21-219)
UL14ND40	N-terminus shortened UL14 (41-219)
UL14ND60	N-terminus shortened UL14 (61-219)
UL14ND80	N-terminus shortened UL14 (81-219)
UL14ND120	N-terminus shortened UL14 (121-219)
UL14CD40	C-terminus shortened UL14 (1-189)
UL14K51A	Lys51 replaced with Ala
UL14R60A	Arg60 replaced with Ala
UL14R(60,64)A	Arg60 and -64 replaced with Ala
PM3	Lys51, Arg60 and -64 replaced with Ala
UL14D(51-90)	40 residues (51-90) replaced with Asp-Ile
UL14D(121-180)	60 residues (121-180) replaced with Asp-Ile

Arg60 and Arg64 are important for nuclear relocation of VP26 and UL14 protein in cotransfected cells

In our previous study we showed that deletion of 80 N-terminal residues of UL14 protein greatly decreased the population of cells in which nuclear translocation of VP26 was observed (Yamauchi et al., 2001). To inspect this in further detail, we constructed a number of UL14 mutant protein-expressing plasmids (Table 1). Point mutations were carried out for three basic residues (Lys51, Arg60 and Arg64) that were conserved between the HSV UL14 protein and the Hsp70 family.

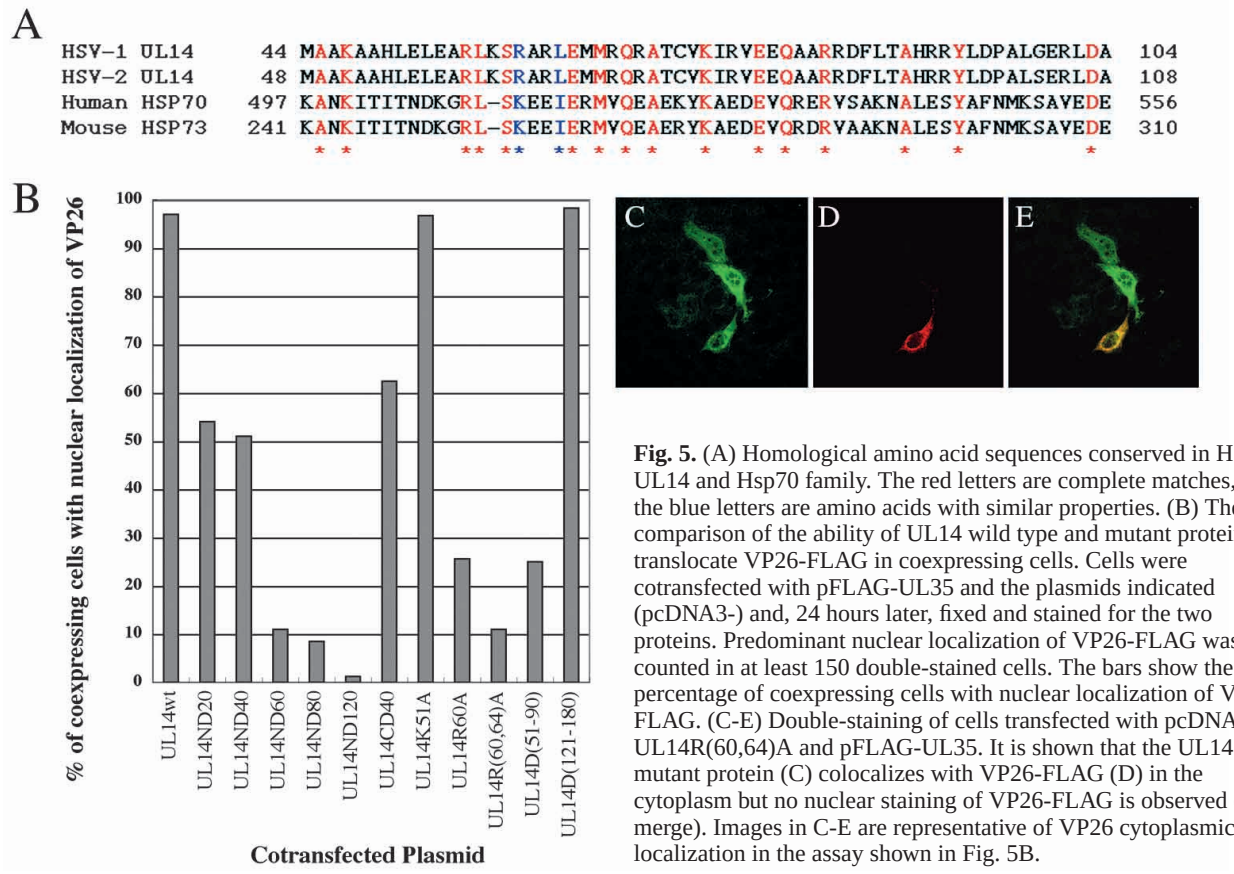

Fig. 5. (A) Homological amino acid sequences conserved in HSV UL14 and Hsp70 family. The red letters are complete matches, and the blue letters are amino acids with similar properties. (B) The comparison of the ability of UL14 wild type and mutant proteins to translocate VP26-FLAG in coexpressing cells. Cells were cotransfected with pFLAG-UL35 and the plasmids indicated (pcDNA3-) and, 24 hours later, fixed and stained for the two proteins. Predominant nuclear localization of VP26-FLAG was counted in at least 150 double-stained cells. The bars show the percentage of coexpressing cells with nuclear localization of VP26-FLAG. (C-E) Double-staining of cells transfected with pcDNA3-UL14R(60,64)A and pFLAG-UL35. It is shown that the UL14 mutant protein (C) colocalizes with VP26-FLAG (D) in the cytoplasm but no nuclear staining of VP26-FLAG is observed (E, merge). Images in C-E are representative of VP26 cytoplasmic localization in the assay shown in Fig. 5B.

Pre-existing and newly constructed plasmids were cotransfected with pFLAG-UL35, a plasmid that expresses a version of VP26 tagged with a FLAG at its C-terminus. Twenty-four hours later, cells were fixed and double-stained for UL14 protein and VP26. In each cotransfection assay, at least 150 double-stained cells were quantified for strong nuclear fluorescence of VP26 in order to compare the translocational properties of the UL14 mutant proteins.

Fig. 5B shows that UL14ND40 was able to translocate VP26 in 50% of cells, whereas UL14ND60 translocated VP26 in only about 10% of cells. UL14ND120 has only about half as many amino acids as the wild-type protein and showed almost no translocational ability. UL14K51A showed almost equal translocational ability compared with wild-type protein, whereas UL14R60A exhibited 26% efficiency in translocation of VP26. UL14R(60,64)A, a double point-mutated UL14 protein (Arg60 and -64 converted to Ala), relocated VP26 into the nucleus only in about 10% of cells; the same efficiency as a mutant protein that lacked 60 N-terminal residues. Fig. 5C-E shows cells expressing UL14R(60,64)A and VP26-FLAG. Interestingly, UL14D(121-180), which lacked 60 amino acids in a region non-homologous to Hsp70 exhibited almost no difference in translocation abilities compared with wild-type UL14 protein. In contrast, UL14D(51-90), which lacked 40 residues in the homologous region, showed considerable defect in the translocational ability (25% effeiciency). These results supported the idea that the Hsp70-homologous region of UL14 protein was important for its translocational function of VP26 in coexpressing cells.

Transiently- and constitutively-expressed UL14 protein can enhance the activity of newly-synthesized firefly luciferase

In the absence of viral proteins, UL14 protein could increase the activity of firefly luciferase. The folding of newly synthesized luciferase in an in vitro assay depends to a great extent (70%) on the chaperones Hsp70 and Hsp40 (Frydman at al., 1994). Therefore, if, when overexpressed, UL14 protein could increase luciferase activity, then it is probably a molecular chaperone-like protein, or perhaps a cochaperone-like protein of cellular HSPs.

HEp-2 cells were transfected with a luciferase-expressing vector pGL3-p and with a control vector pcDNA3 or pcDNA3-UL14 and assayed for luciferase activity 24 hours later. When luciferase activity was moderate in cells transfected with the control vector, cells expressing UL14 protein had higher (140-170%) relative luciferase activity (data not shown). When control cells showed low levels of luciferase activity to begin with, UL14 protein's existence increased luciferase activity even more. This was found to be dose-dependent, for when the transfected pcDNA3-UL14 was increased to fourfold the initial dose, luciferase activity rose to nearly 14-fold (Fig. 6A). We presumed that this resulted from a higher efficiency in folding, meaning a larger number of luciferase molecules being folded to their native state, thus yielding higher activity. Apparently, low levels of luciferase activity in control cells resulted from insufficient folding of newly synthesized luciferase by cellular chaperones such as Hsp70. In such a case, we thought it was possible for UL14 protein to enhance the relative activity of luciferase. On the other hand, high luciferase activity in control cells suggested that folding by cellular chaperones had taken place sufficiently. Therefore, a smaller margin was allowed in the increase of luciferase activity in the presence of UL14 protein.

To evaluate the amount of proteins expressed in these assays, identical samples were used in a western blot to detect expression levels of luciferase and UL14 protein. As shown in Fig. 6A and B, amounts of expressed luciferase were almost the same contrary to the difference in relative activity. This suggested that overexpression of the UL14 protein did not affect the level of expression of luciferase but did enhance the folding of newly synthesized luciferase substrates. We next compared the activity of luciferase expressed on its own in HEp-2 and 14/HEp-2 cells or in Vero and 14/Vero cells. The experiment retrieved similar results (Fig. 6B). Both 14/HEp-2 and 14/Vero cells exhibited higher levels of luciferase activity compared with the parent cell lines: in the former, activity was as high as 160%, and the latter, 500%. Once again, the total amount of luciferase was constant.

The existence of UL14 protein compensates for a part of the loss of Hsp70 chaperone function induced by transfection of antisense oligomers

In the experiments above, we could only estimate how much luciferase was actually folded properly by Hsp70, for this itself seemed to depend on the vital activity of the cell culture. In order to examine the enhancement of luciferase activity by UL14 protein in a cell culture system, which is reduced in functional cellular Hsp70, we introduced an antisense oligonucleotide (5′-CGCGGCTTTGGCCAT-3′) that is complementary to the initiation codon and four downstream codons of the human Hsp70 mRNA, which therefore inhibit the synthesis of Hsp70 when taken into the cell. As controls, the corresponding sense oligomer (5′-ATGGCCAAAGCCGCG-3′) and nonsense oligomer (5′-CGGGTATGCTTCGCC-3′) were used (Wei et al., 1995).

In preliminary experiments, we transfected HEp-2 cells with 0.1 μg pGL3-p alone, or with 0.1 μg pGL3-p and 0.5 μM of sense/antisense oligomer. Twenty-four hours later, the activity of luciferase in antisense-oligomer-transfected cells was significantly lower than that in cells transfected with the sense oligomer. There was no difference between sense- and nonsense-oligomer-transfected cells (data not shown). This outcome suggested that luciferase activity was hindered in antisense-treated cells due to a blockage of Hsp70 synthesis resulting in a lower folding efficiency of newly produced luciferase molecules. Judging from this, we decided to use the same method to observe the effects of constitutive UL14 protein expression on luciferase activity in antisense-treated 14/HEp-2 cells.

Exponentially growing HEp-2 and 14/HEp-2 cells were transfected with 0.1 μg pGL3-p and 0.5 μM of sense/antisense oligomer, or HEp-2 cells were transfected with 0.5 μM of antisense oligomer, 0.1 μg pGL3-p and 0.25 μg pCDNA3-UL14, -UL14R(60,64)A, or -UL14D(51-90), and luciferase activity was measured 24 hours later. In antisense-transfected HEp-2 cells, the activity of luciferase was decreased to about 40% of control (sense oligomer-treated) cells. However, in antisense-transfected 14/HEp-2 cells, activity was only restrained to 60% of control cells. In addition, HEp-2 cells

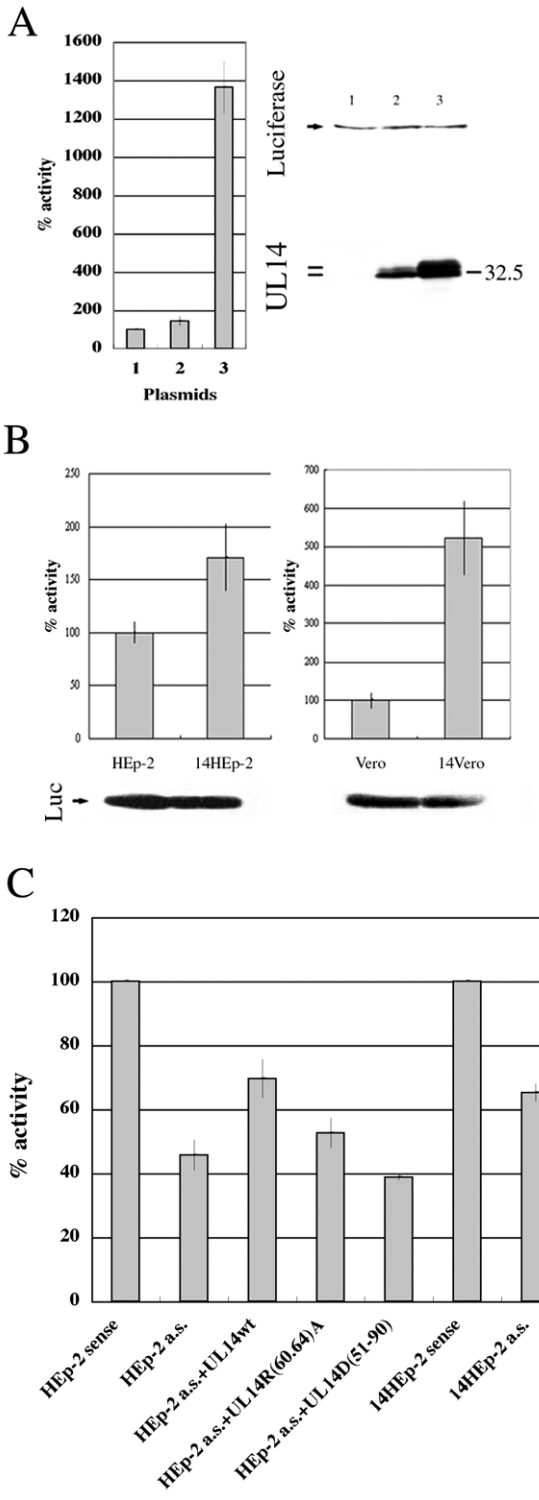

Fig. 6. The presence of UL14 protein increases luciferase activity in a dose-dependent manner when cells yield low luciferase activity. HEp-2 cells were cotransfected with pGL3-p (0.25 μg) and pcDNA3-UL14 (1, 0 μg; 2, 0.50 μg; 3, 1.0 μg) and an empty vector pcDNA3, to a total of 1.25 μg DNA per assay. The results are shown as the percentage of relative luciferase activity. The increase in the expression of UL14 protein correlates with the rise in luciferase activity (up to 14-fold). Each assay was done in triplicate, and each point represents the average±s.d. of three experiments. The same samples were subjected to western blotting and detected for luciferase showing that the level of protein is almost constant contrary to its rise in activity. (B) Luciferase activity is increased in cell lines expressing UL14 protein without a change in the level of luciferase protein. HEp-2, 14/HEp-2, Vero and 14/Vero cells were each transfected with 0.5 μg of luciferase-expressing plasmid pGL3-p and luciferase activity was measured 24 hours later. In each cell line, constitutive expression of UL14 protein increased luciferase activity. Luciferase activity was measured as in A. Western blots show that the amount of expressed luciferase is almost unchanged. (C) UL14 protein existence substitutes for the loss in luciferase activity induced by transfection with antisense oligomer to cellular Hsp70 mRNA. HEp-2 and 14/HEp-2 cells were cotransfected with: (1) 0.5 μM of either sense or antisense (a.s.) oligomers and 0.1 μg pGL3-p; (2) 0.5 μM of anti-sense oligomer, 0.1 μg pGL3-p and 0.25 μg pCDNA3-UL14, -UL14R(60,64)A, or -UL14D(51-90). 14HEp-2 cells were cotransfected with 0.5 μM of either sense or antisense oligomers and 0.1 μg pGL3-p. Luciferase activity was assayed as above and relative activity was compared. The results show that the drop in luciferase activity on transfection of antisense oligomers is less in 14/HEp-2 cells (35%) than in HEp-2 cells (55%). In addition, luciferase coexpressed with UL14 mutant proteins showed lower activity, suggesting that UL14 wt protein compensates for the loss of cellular chaperone activity. Each assay was done in triplicate, and each point represents the average±s.d. of three experiments.

coexpressing luciferase with UL14 wt protein showed higher activity than those expressing UL14 mutant proteins (Fig. 6C). This implied that the loss of chaperone activity induced by antisense oligomers is most compensated when these cells were overexpressed with the full-length UL14 protein.

Discussion

The present study suggests that UL14 protein of HSV-2 has HSP-like properties. This was indicated not only by the protein's striking change in intracellular localization on reaction to stressful environments, but also by the enhancing effect on the enzymatic activity of newly synthesized firefly luciferase. What aroused interest was our previous observation that UL14 protein can mutually translocate other HSV-2 proteins into the nucleus of coexpressing cells: namely VP26, the UL35 product, which is the minor capsid protein; and the UL33 product, which is known to play a role in DNA cleavage and packaging. Another protein was UL17, whose population of cells with nuclear localization rose considerably under the presence of UL14 (Yamauchi et al., 2001). These translocations suggested that UL14 may be a molecular chaperone-like protein, leading to the idea that UL14 protein's intracellular localization might be affected by stress such as heat shock.

Our trials resulted in the rapid nuclear and nucleolar translocation of UL14 protein after a brief (<30 minutes) heat shock at 43°C, which could be reversed in a matter of hours when cells were recovered at 37°C. These results were symbolic of an HSP-like feature of UL14 protein. Moreover, UL14 protein localized in nuclei in ATP-depleted and hyperosmotic-shocked cells, further suggesting that the protein functioned as a stress protein in reaction to the surrounding environment. In these cases, UL14 protein localized in DNA-excluding areas of the nucleus (the nucleoli and nuclear matrix) as shown by double staining with propidium iodide.

After a 30 minute heat shock in 14/Vero cells, UL14 protein

colocalized with PML or ND10. ND10 are dynamic structures that are disrupted during mitosis and respond to environmental stimuli including interferon treatment, heat-shock, treatment with heavy metals, and viral infection (Maul et al., 1995; Stuurman et al., 1997; Stuurman et al., 1992). PML and Sp100 are components of ND10 whose proteasome-dependent degradation is induced by HSV-1 regulatory protein Vmw110 (Everett et al., 1999b). ND10 proteins are recruited to replication compartments in HSV-1 infected cells and ND10 or sites in the cell close to ND10 play an important role in the establishment of a productive infection (Maul et al., 1993; Burkham et al., 1998).

In heat-shocked 14/HEp-2 cells, UL14 protein localized at the centromeres of M phase cells and colocalized completely with NuMA. The centrosome, consisting of centrioles and pericentriolar material, is the most common organizer of microtubules in mammalian cells (Archer and Solomon, 1994; Schatten, 1994). Vmw110 is known to interact with centromeres in mitotic and interphase cells in a way similar to its interaction with ND10 (Everett et al., 1999a). Hsp70 protects the centrosome during heat shock (Liang and MacRae, 1997) and, with Hsp27, centrosomes become associated with and co-isolate with the nuclear matrix following heat shock (vanderWaal et al., 1996). The kinetics of the nuclear localization and delocalization of Hsp70 are constant with it having a protective role for the nuclear matrix (RotiRoti et al., 1998). It is possible that the translocation of UL14 protein to the nuclear matrix, ND10 and centromeres is a result of its functions in protecting vital compartments of the cell from heat denaturation.

HSPs, otherwise known as molecular chaperones or stress proteins, are constitutively expressed at normal growth temperatures and have basic and indispensable functions in the life cycle of the cell; most are acidic proteins. Hsp40 and Hsp70 are localized mainly in the cytoplasm at normal growth temperatures and translocate into the nuclei and nucleoli upon heat shock. The isoelectric point of UL14 protein is 5.33 and that of Hsp70 is 5.36 (Genetyx Mac), displaying another similarity between the proteins. Interestingly, these proteins return to the cytoplasm during a recovery period after heat shock, although the mechanism is unclear (Ohtsuka and Hata, 2000). Hsp70 binds to and releases polypeptides concomitant with ATP binding and hydrolysis, and possesses two distinct domains. The N-terminal 44 kDa domain of DnaK (the Hsp70 homologue) is highly conserved among all DnaK members, while the C-terminal 27 kDa domain is more variable and mediates substrate binding. The crystal structure of the substrate-binding domain of DnaK (residues 389-607) comprises a β-structured polypeptide-binding site and a latch-like subdomain of five α-helices at the C-terminal end of the protein (Flaherty et al., 1990; Zhu et al., 1996). Although the α-helical subdomain is not in direct contact with the bound peptide it is thought to work like a lid that facilitates access to the substrate-binding site (Beissinger and Buchner, 1998).

We found a homology between HSV UL14 proteins and the Hsp70 family proteins. The domain with the most homology is located in the N-terminal part (residues 60-74) of HSV-2 UL14 protein and the C-terminal part (residues 509-522) of the 18 kDa peptide-binding domain of human Hsp70. The 18 kDa peptide-binding domain (residues 384-543) contains two four-stranded antiparallel β-sheets and a single α-helix (Kiang and Tsokos, 1998). The homology is located in the centre of the α-helix. The α-helix of Hsp70 is a nine amino acid stretch [(511) S-K-E-D-I-E-R-M-V (519)], and the homology with UL14 is [(63) S-R-X-X-L-E-X-M-X (71)]. Thus five out of nine residues are homologous; the Lys of Hsp70 and the Arg of UL14 may be of particular importance for chaperone function of both proteins. Wang et al. indicated that the Hsc70 peptide-binding domain appears to be very stable and relatively independent of the rest of the molecule (Wang et al., 1993). The binding site may be located on the α-helix, because the potent immunosuppressor 15-deoxy-spergualin, a synthetic antitumor agent known to bind to Hsc70, could be carried by such a site. In this report, Arg60 and -64 of the HSV-2 UL14 protein, which are included in the sequence (60) R-L-K-S-R (64), were shown to be important for the relocation of the HSV-2 UL14 protein itself and VP26 into the nucleus of coexpressing cells. This sequence is located at the N-terminal side of the α-helix of the peptide-binding domain of Hsp70. In terms of homological sequences, we have also searched for other molecular chaperones. There seemed to be a number of matches in the amino acid alignments with the chaperonin Hsp60, and Hsp27. Unfortunately, these matches were not as convincing as the homology of UL14 protein with Hsp70. Nevertheless, it is worth noting that the matches were found in the N-terminal area in the vicinity of the Hsp70-homologous region of UL14 protein. The small HSP and α-crystallin family consists of 12-43 kDa proteins that assemble into multimeric structures and contain a conserved C-terminal region termed the α-crystallin domain (Fink, 1999). Alhough the molecular mass of UL14 is 32 kDa, there was no conserved sequence in the C-terminus relative to such a domain.

When cells were subjected to ATP depletion, Hsp70 was shifted towards its aggregated forms in a manner similar to that observed after heat treatment (Angelidis, 1999). The nuclear aggregation and translocation of UL14 protein upon ATP-depletion was very similar to this. Although UL14 protein does not have an ATPase domain, according to sequence analysis, its change in intracellular localization suggests involvement of an ATP-dependent mechanism. UL14 protein translocated to the nucleus also by hyperosmotic shock. Although immunofluorecence showed that the nuclear staining-patterns differed in heat-shocked, ATP-depleted or osmotic-shocked cells, the protein clearly exhibits stress-induced changes. It was shown that Hsp33 chaperone function is regulated at the post-translational level by the redox conditions that distinguish it from all other known molecular chaperones (Jakob et al., 1999). It will be of considerable interest to research the regulation mechanism of UL14 protein in HSV-infected cells.

Finally, the increase in luciferase activity in the presence of UL14 protein, whether in a transiently- or constitutively-expressed state, suggested that the protein positively affected the folding of newly synthesized luciferase substrates. Higher luciferase activity was achieved by increasing the enzymatic activity of luciferase and not by a change in expressed amounts of luciferase. It is known that the enzymatic activity of luciferase during recovery from thermal inactivation is highly dependent on chaperone activity, and overexpression of Hsp70 alone is sufficient to enhance reactivation of heat-denatured luciferase activity during recovery at 37°C (Michels et al., 1997). During recovery from heat denaturation at 45°C, the

level of luciferase activity in UL14-protein-expressing cell lines was no greater than that in non-expressing cells (data not shown). Although UL14 protein may contribute to thermotolerance of the cell, it was not used in the folding process after heat denaturation. A possible explanation is that UL14 protein becomes predominantly nuclear upon heat shock and is slower than Hsp70 in returning to the cytosol upon recovery. This means that the protein is not in contact with luciferase substrates in the cytoplasm for at least a few hours, unlike Hsp70, which can localize in the cytoplasm even after heat shock. Overall, we conclude that UL14 protein is an HSP-like protein.

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