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Protection against heat and staurosporine mediated apoptosis by the HSV-1 US11 protein

E. Javouhey [a], B. Gibert [a], A.-P. Arrigo [a], J.J. Diaz [b], C. Diaz-Latoud [a,*]

[a] Laboratoire Stress, chaperons et mort cellulaire, Université de Lyon, Lyon, F-69003, France; Université Lyon 1, Lyon, F-69003, France; CNRS, UMR5534, Centre de génétique moléculaire et cellulaire, Villeurbanne, F-69622, France
[b] Laboratoire domaines nucléaires and Laboratoire pathologies, Université de Lyon, Lyon, F-69003, France; Université Lyon 1, Lyon, F-69003, France; CNRS, UMR5534, Centre de génétique moléculaire et cellulaire, Villeurbanne, F-69622, France

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ABSTRACT

US11 protein, one of herpes simplex virus type 1 (HSV-1) true late gene products, plays a role in the virally induced post-transcriptional control of gene expression. In addition, US11 expression also interferes with the cellular response to HSV-1 infection that can lead to apoptosis. We have previously shown that US11 expression enhanced the recovery of cellular protein synthesis and increased cell survival in response to thermal stress. Since heat shock can activate apoptosis, we tested for a possible anti-apoptotic behavior of US11. Here, we show that, in HeLa cells, US11 expression strongly reduced heat induced apoptosis, a phenomenon independent of Hsp expression and characterized by a delayed cytochrome *c* efflux from mitochondria and reduced caspase 3 activation. Moreover, US11 expression also protected against staurosporine induced apoptosis. Hence, our results favor an anti-apoptotic activity of US11 polypeptide that appears to be located at the level of mitochondria or upstream signaling pathways.

Introduction

Apoptosis or programmed cell death, a highly regulated process activated by various cell metabolism disturbing stimuli, is characterized by membrane blebbing, chromatin condensation, nuclear fragmentation and finally the formation of apoptotic bodies (Green, 1998). Apoptotic execution is associated with activation of a family of cysteine proteases known as caspases, which are broadly categorized as initiator (caspase-2, -8, -9, -10) or executioner caspases (caspase-1, -3, -4, -6, -7), that cleave specific proteins leading to the typical hallmarks of apoptosis (Earnshaw et al., 1999; Lavrik et al., 2005). Two main activation cascades for apoptosis induction have been identified. One is induced through death receptors such as Fas/CD95, TNFα (Baetu and Hiscott, 2002; Krammer, 2000) recruiting adapter proteins which in turn bind and directly activate caspases (Nagata, 1997). In the second pathway, through the action of pro-apoptotic Bcl-2 family of polypeptides, mitochondrial membrane permeability is enhanced and cytochrome *c* and other pro-apoptotic molecules are translocated from the mitochondria to the cytosol (Adams and Cory, 1998). After cytochrome *c* release, apoptosis is initiated by the formation of apoptosome, which triggers the caspase activation cascade (Li et al., 1997).

Transient exposure of cells to a mild heat shock activates cellular stress response and results in synthesis and accumulation of heat shock proteins (Hsps). These evolutionary conserved proteins render cells thermotolerant, resistant to subsequent lethal insults (Georgopoulos and Welch, 1993), and protect them from the cytotoxic effects induced by aggregated proteins (Hendrick and Hartl, 1993). They also function as key regulators in the control of apoptosis (Beere et al., 2000; Bruey et al., 2000; Mehlen et al., 1996; Park et al., 2001).

Severe heat shock can cause cell viability loss and the resulting cell death can occur through two morphologically and biochemical distinct pathways: either necrosis, followed, *in vivo,* by an inflammatory response (Harmon et al., 1990), or apoptosis with the involvement of either the extrinsic or the intrinsic canonical pathways (Tibbles and Woodgett, 1999; Zhao et al., 2006). The initial trigger for activation of apoptosis after heat shock is not clearly understood but as prolonged activation of the UPR can lead to apoptosis it might be linked to the unfolded protein response (UPR) (Schroder and Kaufman, 2005). Recent studies suggest that hyperthermia mediated cell death is

Abbreviations: DMEM, Dulbecco's modified Eagle's medium; DEVD-AFC, Asp-Glu-Val-Asp-7-amino-4-trifluoromethylcoumarin; ER, Endoplasmic Reticulum; FADD, FAS-associated death domain; GRP94, Glucose-Regulated protein 94; HSV-1, herpes simplex virus type 1; IETD-AFC, Ile-Glu-Thr-Asp-7-amino-4-trifluoromethylcoumarin; JNK, c-Jun NH_2 terminal kinase; LEHD-AFC, Leu-Glu-His-Asp-7-amino-4-trifluoromethylcoumarin; MOMP, mitochondrial outer membrane permeabilization; TNFα, tumor Necrosis Factor α.

* Corresponding author. Laboratoire stress, chaperons et mort cellulaire, CNRS UMR 5534, Centre de Génétique Moléculaire et Cellulaire, Université Claude Bernard, Lyon 1, 43, Bd du 11 Novembre, 69622 Villeurbanne Cedex, France. Fax: +33 472 43 26 85.
E-mail address: chantal.diaz@univ-lyon1.fr (C. Diaz-Latoud).

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independent of any of the known initiator caspase and therefore might be induced through a specific pathway involving a novel apical protease. This unidentified protease induces mitochondrial outer membrane permeabilization (MOMP), cytochrome *c* release from the mitochondria inter-membrane space to the cytoplasm and caspase 3 activation (Milleron and Bratton, 2006).

Apoptosis is also a mechanism of host cell defense against viral infections. Consequently, many viruses have developed mechanisms to block the premature death of the infected cells. Interestingly, HSV-1, a large DNA virus coding for at least 80 genes could be considered as a prototype of "anti-apoptotic" virus encoding several well defined proteins that are able to counteract the wide diversity of HSV infection-triggered cellular apoptosis albeit by mechanisms that remain still largely unknown for most of them (for a review see (Nguyen and Blaho, 2007)). US3, US5, ICP6 and LAT RNA are HSV gene products that are able to counteract apoptosis in the absence of other viral functions (Jerome et al., 1999; Langelier et al., 2002; Perng et al., 2000). Among these polypeptides, the one with the best characterized anti-apoptotic activity is the US3 protein kinase (Nishiyama and Murata, 2002). US3 kinase blocks apoptosis at a pre-mitochondrial stage by degradation or posttranslational modification of BAD, a pro-apoptotic member of the Bcl-2 family (Munger et al., 2001) or by suppression of JNK activation (Murata et al., 2002). US3 also exerts anti-apoptotic effect downstream mitochondria and this inhibition is dependent upon US3 catalytic activity (Ogg et al., 2004) and blocks the proteolytic cleavage of procaspase 3 (Benetti and Roizman, 2007).

US11 is an RNA-binding protein, post-transcriptional regulator of gene expression (Attrill et al., 2002; Bryant et al., 2005; Diaz et al., 1996). US11 interacts with several different cellular proteins such as human ubiquitous kinesin heavy chain (uKHC) (Diefenbach et al., 2002), homeodomain interacting protein kinase 2 (HIPK2) (Giraud et al., 2004), double-stranded RNA-dependent protein kinase (PKR) and a dsRNA-independent protein activator of PKR (PACT) (Cassady and Gross, 2002; Peters et al., 2002). US11 has been reported as a potent inhibitor of PKR activation through binding to dsRNA (Khoo et al., 2002) or through direct interaction with PKR in the context of viral infection (Cassady and Gross, 2002) and therefore could interfere with the PKR mediated host cell responses. PKR expression is stimulated by type 1 interferon during the cellular interferon-inducible antiviral response. This serine-threonine kinase appears to play a primary role in mediating the antiviral activities of infected cells through activation of the FADD/caspase 8 death-signaling pathway (Balachandran et al., 2000). PKR also plays an important role during ER stress-induced cell death and is essential for efficient induction of the stress protein GRP94 (Ito et al., 2007). Finally, US11 has been recently shown to also counteract the activity of the 2′–5′ oligoadenylate synthetase (OAS), a cellular protein critical for host cell defense (Sanchez and Mohr, 2007). All these observations suggest that US11 might be involved in the viral response against host cell defense and in particular in the inhibition of apoptosis induced by viral infection although none of these studies allow to clearly deciphering the molecular mechanisms by which this could occur. Furthermore, these studies did not address directly the question of whether HSV could counteract apoptosis induced by a non-viral stress – like heat shock – that is known to induce viral reactivation in humans.

We have previously shown that after heat shock, US11 exacerbates the recovery of cellular protein synthesis and provides an increased cell survival to thermal stress (Diaz-Latoud et al., 1997). Because severe thermal stress induces apoptosis and is also one of the most powerful inductor of HSV reactivation, we hypothesized that US11 mediated protection against the deleterious effects of heat stress could result from its ability to counteract heat induced-apoptosis. Here, we present evidence that in US11 expressing cells exposed to heat shock, caspase 3 activation is delayed and cytochrome *c* efflux from mitochondria strongly reduced. This suggests a yet undescribed anti-apoptotic role for this viral protein at the level of mitochondria or upstream signaling pathways that could be relevant for successful HSV replication following reactivation by the uncontrolled increase of cell temperature in infected humans.

Results

US11 protects cells against heat induced apoptosis

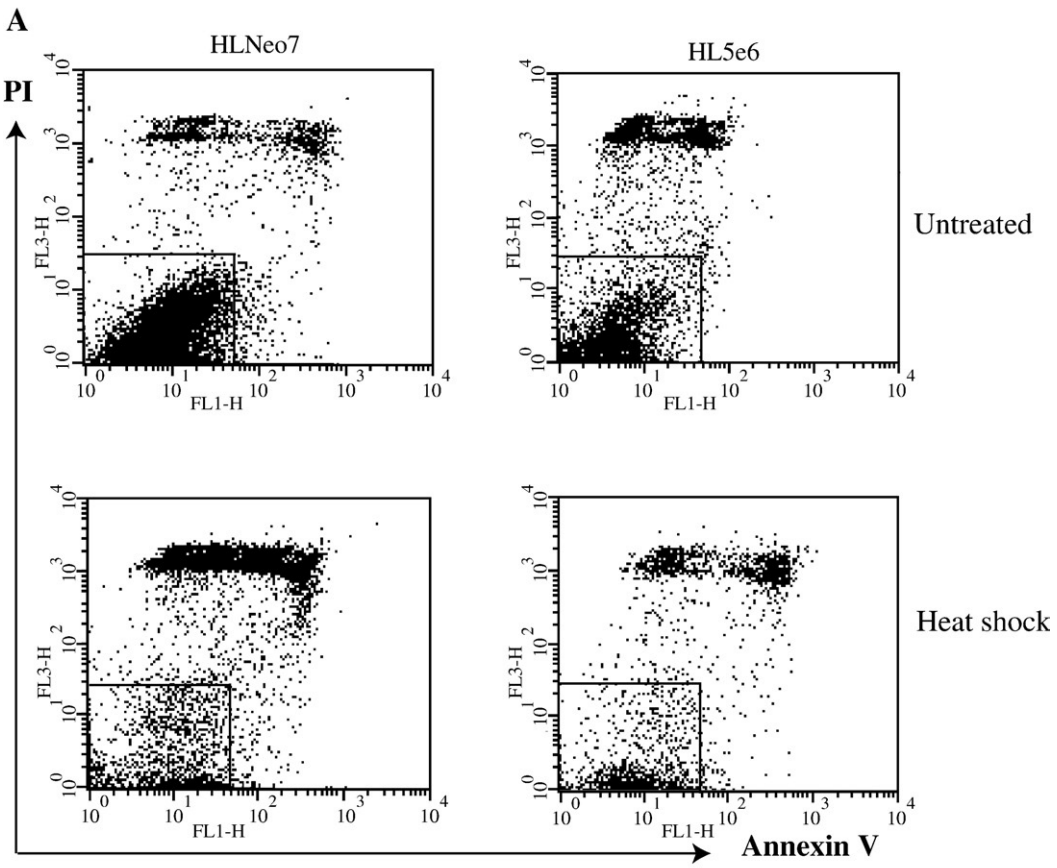
The resistance to heat induced apoptosis was analyzed in the previously characterized HeLa cells constitutively expressing US11, HL5e6 (Simonin et al., 1995). HL5e6 and control HLNeo7 cells were heat treated for 1 h at 44 °C and allowed to recover for 3 h. The extent of heat induced apoptosis was then determined by measuring the level of externalization of phosphatidylserines (PS), an event which is observed during maximal apoptosis and that can be visualized by the binding of (FITC)-conjugates Annexin V to non-permeabilized cells. Fig. 1A presents two-dimensional FACScan analysis of propidium iodide (PI) / Annexin V staining. Quantitative analysis (Fig. 1B) showed that, after heat shock treatment, the percentage of viable HLNeo7 cells (PI and Annexin V negative cells) dropped from 82% to 52% of the total cell population whereas the decrease was far less intense (77% to 73%) in US11 expressing cells. Statistical analysis demonstrated the significance of the differences in the percentages of viable cells observed between control and US11 expressing cells. These results strongly suggest that the expression of US11 protein protects HeLa cells against heat induced apoptosis.

US11 reduces caspase 3 activation after heat shock

The processing of the executioner caspase 3 is an essential event to complete the destructive phase of apoptosis (Slee et al., 1999). Therefore, to further characterize US11 protein function in HeLa cells during heat shock, we next analyzed whether this polypeptide could modulate the heat induced activation of caspase 3. To render the results more robust, these experiments were performed using two different clones of HeLa cells expressing constitutively different levels of US11, HL5e6 clone expressing 50% more viral protein than HL5a1 cells as already described (Diaz-Latoud et al., 1997). An immunoblot analysis of the 32 kDa procaspase 3 processing, in HeLa cells expressing or not US11, after a heat shock treatment (1 h at 44 °C) and during recovery at 37 °C is presented in Fig. 2A. The analysis of the pixel density of procaspase 3 band showed a strong decrease in the level of procaspase 3 in control cells after heat shock whereas no decrease was observed in US11 expressing cells. As expected the 17 kDa caspase 3 processed fragment was not detected in untreated cells. This fragment was clearly detected in HLNeo7 cells 2 h after the end of heat shock and its amount reached a maximum level after a 4 h recovery period. Conversely, this fragment was not detected in both US11 expressing cells after a 2 h recovery period and was barely detectable after 8 h of recovery in HL5a1 cells.

In order to monitor more finely this phenomenon, heat induced caspase 3 activity was then monitored by cleavage of fluorescent DEVD-AFC substrate analysis (Fig. 2B). As expected, heat shock induced an important increase of caspase activity in HLNeo7 control cells that reached up to a 10-fold activation after 4 h of recovery (Fig. 2B, black bars). Conversely, heat shock did not induced a significant caspase 3 activation in both US11 expressing cell lines whatever was the duration of recovery period (2, 4 and 6 h) (Fig. 2B, grey bars).

To determine if the delay in caspase 3 activation observed in US11 expressing cells was not a consequence of a cellular adaptation to the constitutive presence of US11, we performed the same type of experiments in HeLa cells transiently expressing US11. HeLa cells transfected with either an US11 expressing vector or a control vector were submitted to a one-hour heat shock at 44 °C 36 h after



B

	HLNeo7	HL5e6
Untreated	80.47 % +/- 1.53	77.3 % +/-1.07
Heat shock	52.43 % +/- 2.63	72.99 % +/- 0.68

Percentage of viable cells

Fig. 1. US11 protein reduces heat induced apoptosis. HLNeo7 control and HL5e6 US11 expressing cells, treated for 1 h at 44 °C, were harvested 3 h after the heat shock treatment. (A) Cells were then stained with Annexin V and propidium iodide and analyzed as described in Materials and methods. Data are representative of one out of three experiments with comparable results. (B) Lower left square: Annexin V and PI negative cells quantification from panel A. Values represent mean ± S.D of three independent experiments. Student's *t* test analysis demonstrated significance $P=0.0035$ of the differences between staurosporine treated HLNeo7 and HL5e6 cell lines.

transfection and procaspase 3 processing was evaluated after 4 h of recovery at 37 °C. This experiment clearly showed that caspase 3 activation was 2-fold higher in cells transfected with the control vector than in cells transfected with the US11 expressing vector (data not shown). It was then concluded that the anti-apoptotic activity of US11 described here did not result from an adaptation of the cells to a prolonged expression of this viral protein.

US11 protein precludes heat shock induced cytochrome c release induced by heat shock

As most apoptotic inducers, heat shock induces the release of cytochrome *c* from the mitochondria inter-membrane space to the cytoplasm (Samali et al., 2001). We therefore determined the level of cytochrome *c* efflux from mitochondria in control HLNeo7 and US11 expressing HL5e6 cells either immediately after heat shock or after 3 h of recovery (Fig. 3A). Cells were heat shock treated for 1 h at 44 °C and the intensity of cytochrome *c* release was determined after cell fractionation as described in the Materials and methods section. As control of mitochondria integrity after cell fractionation, cytochrome *c* was undetectable in the supernatant fraction of untreated cells. A high level of cytochrome *c* was detected in the supernatant of control cells immediately after the heat treatment whereas the level found in HL5e6 cells was very low. Three hours after heat shock the level of cytochrome *c* detected in the cytosol of control cells was less important than immediately after heat shock suggesting a degradation of this polypeptide. At the same time period in HL5e6 cells, the level of cytosolic cytochrome *c* began to increase although it never reached that observed in control cells immediately after heat stress. These results lead to the conclusion that US11 protein dramatically delayed the cytochrome *c* efflux from mitochondria induced by heat shock.

The canonical mitochondria-dependent apoptotic pathway leads to the caspases cascade initiation resulting from the activation of

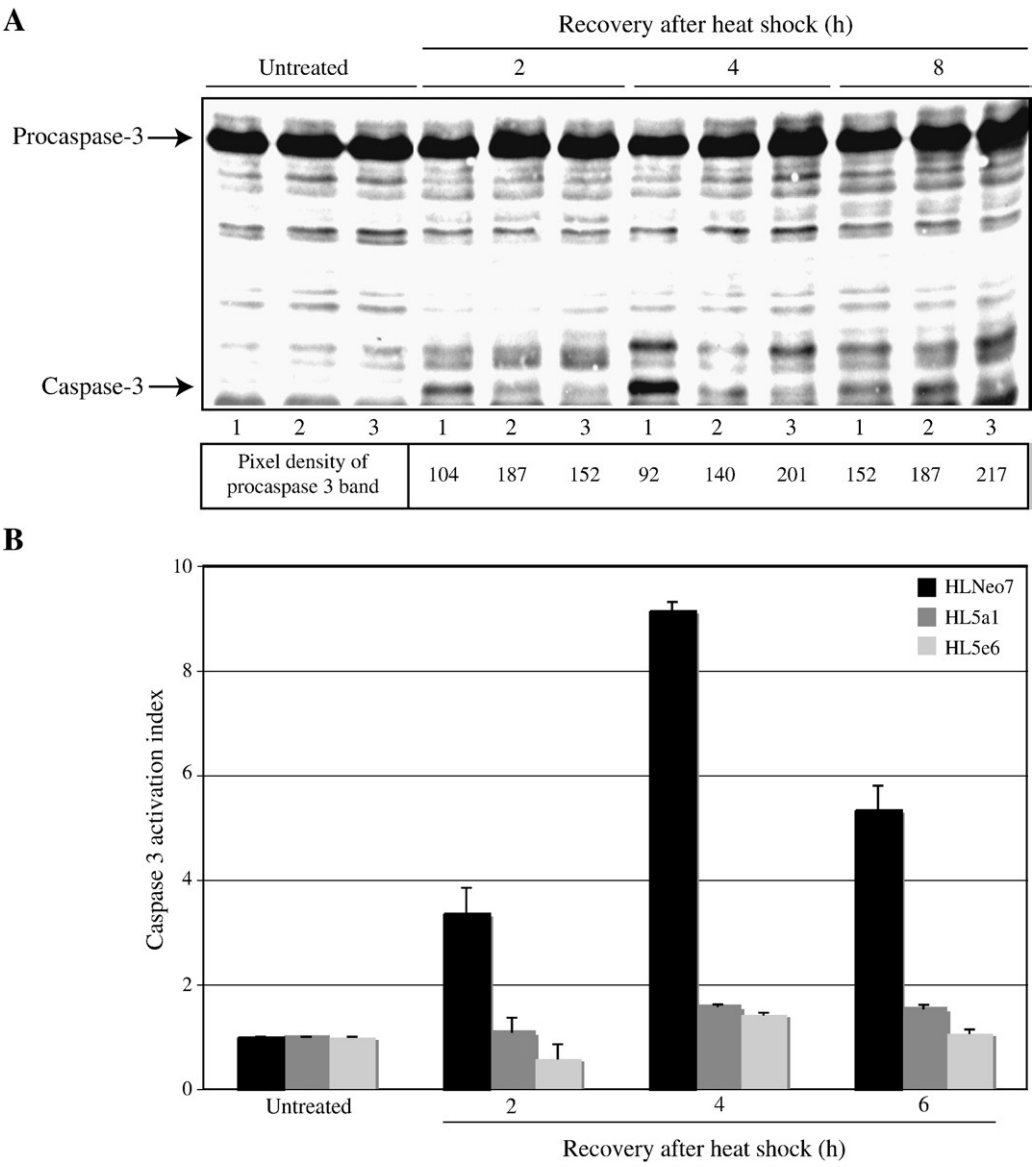


Fig. 2. US11 protein reduces caspase 3 activation after heat shock. (A) Procaspase 3 processing analysis. HLNeo7 control cells (lanes 1) or HL5a1 and HL5e6 US11 expressing cells (lanes 2 and 3 respectively) were heat shock treated for 1 h at 44 °C and allowed to recover for 2 to 8 h before analysis. Cell extracts were analyzed in immunoblots probed with anti-caspase 3 antibody. The procaspase 3 form was quantitated. (B) Caspase 3 activation analysis. Control HLNeo7 and US11 expressing HL5a1 and HL5e6 cells were treated as described above in (A) and allowed to recover for 2 to 6 h. Activity of DEVD-specific caspase was then measured using the fluorescent substrate DEVD-AFC as described in Materials and methods. Activation index was determined as the ratio between activities in extracts of treated cells to that measured in extracts of untreated cells. Columns represent the mean and error bars represent the standard deviation of three experiments. Note the protective activity of US11 protein observed 4 h after heat shock that correlates with the inhibition of procaspase 3 cleavage shown above in (A).

caspase 9 as a consequence of the formation of the apoptosome machinery which consists of procaspase 9, Apaf-1 and translocated cytochrome *c* (Li et al., 1997). Therefore, as we observed a reduced cytochrome *c* efflux from mitochondria in heat shock treated US11 cells, we next analyzed the level of procaspase 9 processing by western blot analysis (Fig. 3B) or by a fluorometric assay (Fig. 3C). This was done using HLNeo7 and US11 expressing cells, HL5e6 and HL5a1, exposed to heat shock. Cells were exposed or not to heat shock for 1 h at 44 °C and analyzed after 2 or 4 h of recovery at 37 °C. As shown in Fig. 3B, in control HLNeo7 cells exposed to heat shock, a slight decrease in the level of procaspase 9 was observed after 4 h of recovery following heat shock suggesting a cleavage. Conversely, no decrease in procaspase 9 level was observed in the US11 expressing HL5a1cells.

As shown in Fig. 3C, in control HLNeo7 cells, heat shock induced a moderate cleavage of the caspase 9 fluorogenic substrate LEHD-AFC in response to heat shock whereas the effect was attenuated in both US11 expressing cells, HL5e6 and HL5a1. It is also intriguing to note that in US11 cells allowed to recover for 6 h after heat shock, the activity of caspase 9 was even lower than the activity observed in untreated cells. These results show that US11 interferes with the weak caspase 9 activation in HeLa cells after heat shock.

US11 protein reduces caspase 8 activation after heat shock

The c-Jun NH_2-terminal kinase (JNK) activation in mammalian cells is an early event in stress induced apoptotic program (Adler et al., 1995). Furthermore JNK activation initiates a FADD-dependent caspase-8 signal transduction pathway leading to programmed cell death (Chen and Lai, 2001). These observations lead us to analyze procaspase 8 processing in HLNeo7 and HL5a1 after 2, 4 or 8 h of

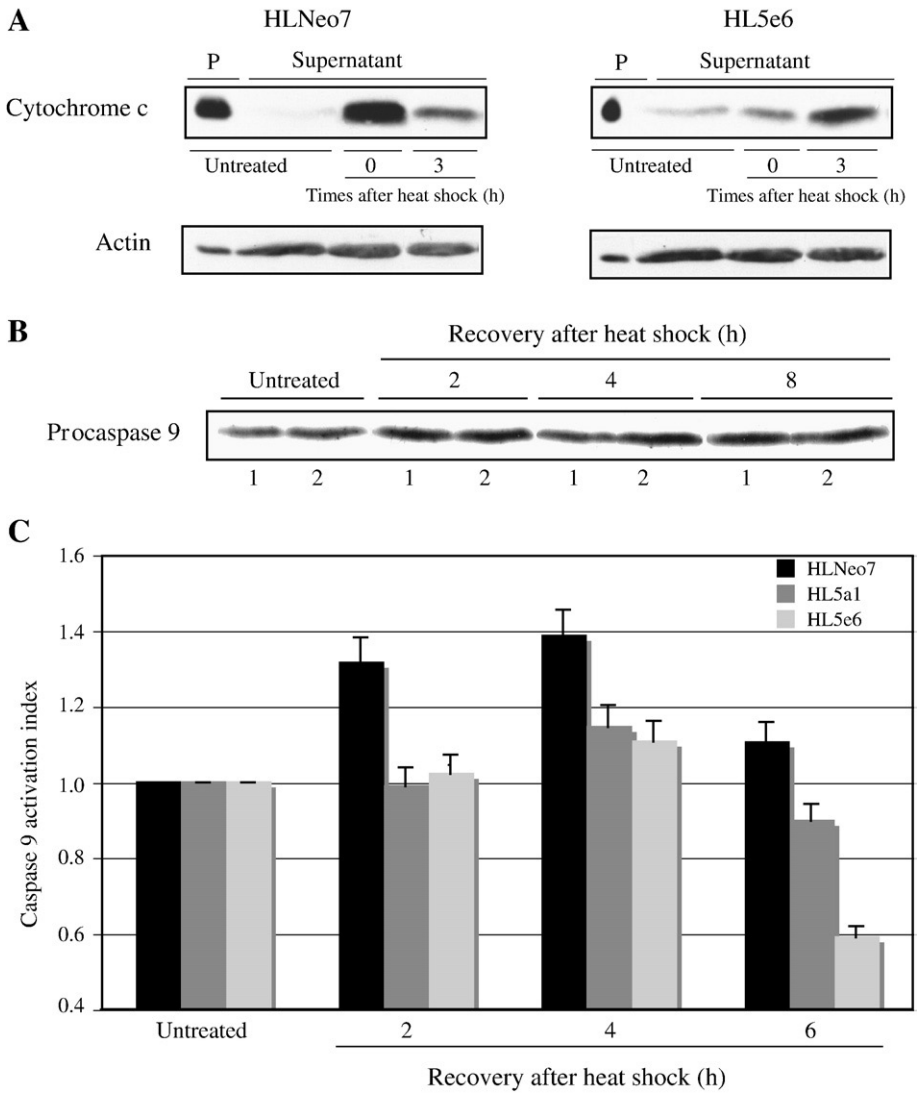


Fig. 3. US11 expression interferes with cytochrome *c* release and with the heat shock induced caspase 9 activation. (A) Cytochrome *c* release analysis. Control HLNeo7 and US11 expressing HL5e6 cells were either kept untreated or heat shock treated for 1 h at 44 °C and allowed or not to recover for 3 h. Cells were then processed and proteins were analyzed in immunoblots as described in Materials and methods. The presence of cytochrome *c* and actin in the different fractions is shown. P: pellet from untreated cells. Supernatant: soluble fraction of untreated cells or heat shock treated cells. Note that US11 protein strongly decreases the release of cytochrome *c* observed in control HLNeo7 cells immediately after heat shock. (B) Procaspase 9 processing analysis. Control HLNeo7 (lanes 1) and US11 expressing HL5a1 cells (lanes 2) were either kept untreated or heat shock treated for 1 h at 44 °C and allowed to recover for 2 to 8 h. Cells extracts were immunobloted with an anti-caspase 9 antibody. (C) Caspase 9 activation analysis. Control HLNeo7 and US11 expressing HL5a1 and HL5e6 cells were treated as described above in (B) and allowed to recover for 2 to 6 h. Caspase 9 activity was measured against fluorometric substrate LEHD-AFC as described in Materials and methods. Activation index was determined as the ratio between the activities in extracts of treated cells to that measured in extracts of non-treated cells. Columns represent the mean and error bars represent the standard deviation of three experiments. Note the protective activity of US11 protein that correlates with the inhibition of procaspase 9 cleavage shown above in (A).

recovery at 37 °C following a 1 h heat shock at 44 °C. As shown in the immunoblot (Fig. 4A), exposure of control HLNeo7 cells to heat shock resulted in a weak but reproducible decrease in the level of the p55 kDa procaspase 8 isoform and a stronger decrease of the p53 kDa isoform as shown by the pixel density analysis. In contrast, in HL5a1 cells the level of these proteins remained unchanged and this even 8 h after the heat shock treatment. Caspase 8 activation was also monitored using a fluorometric assay. US11 expressing, HL5a1 anHL5e6 and control HLNeo7 cells were heat shocked for 1 h at 44 °C and allowed to recover for 2, 4 or 6 h before caspase 8 activation was determined by the IETD-AFC cleavage assay. As shown in Fig. 4B, in control cells, heat shock induced a stimulation of the activity of caspase 8 that reached a maximum 2-fold intensity after 4 h of recovery. In contrast, heat shock did not stimulate caspase 8 activity in both US11 expressing cell lines but rather induced an inhibition of this activity.

US11 protects against apoptosis independently of the presence of the heat shock proteins

Since (i) after heat shock US11 protein enhances protein synthesis recovery and (ii) Hsps are known to protect against apoptosis, we next analyzed whether some of the effects described above could be attributed to an enhanced accumulation of Hsps after heat shock. For this, cells were treated with cycloheximide prior to heat shock treatment in order to inhibit translation during and after heat shock. Control HLNeo7 and US11 expressing HeLa cells were heat shock treated for 1 h at 44 °C in the presence or not of 20 μg/ml of cycloheximide and allowed to recover for 4 h before the immunoblot analysis of procaspase 3 processing was performed. As shown in Fig. 5A, cycloheximide treatment alone did not induce procaspase 3 cleavage in control or US11 expressing cell lines. In contrast, the processing of caspase 3 observed in control HLNeo7 cells exposed to

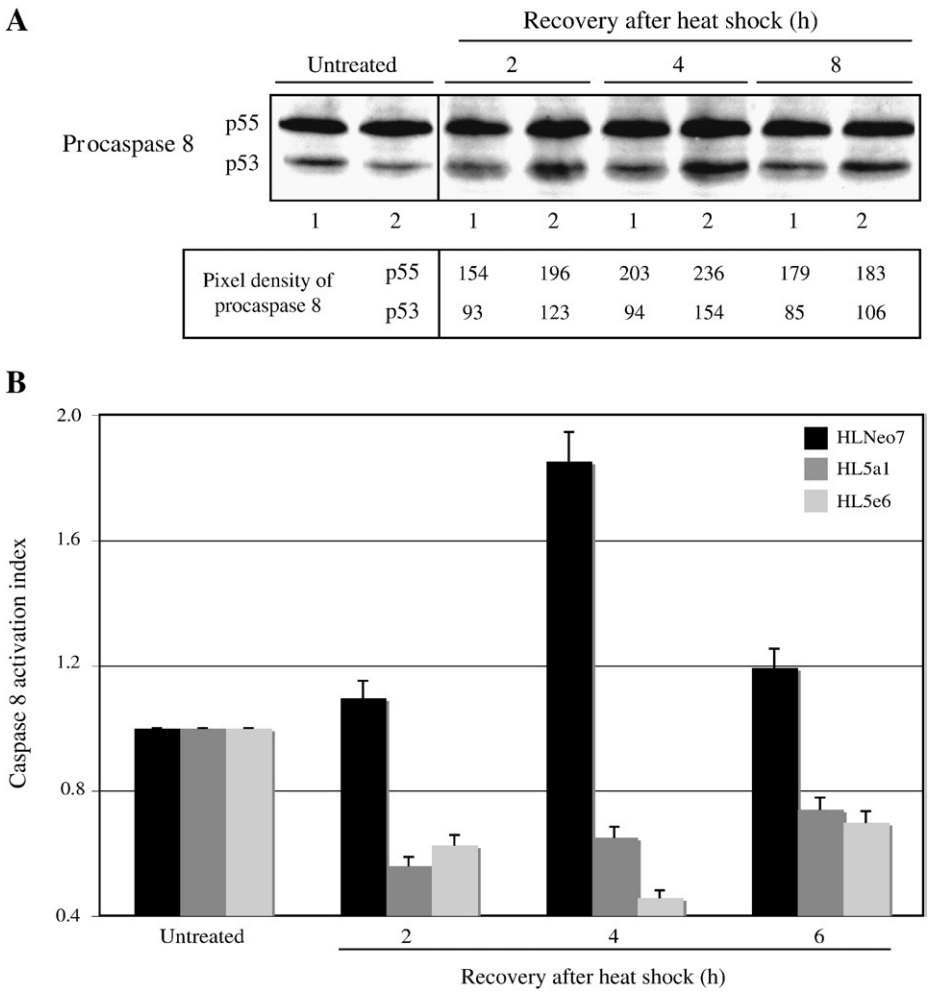


Fig. 4. US11 protein reduces caspase 8 activation after heat shock. (A) Procaspase 8 processing analysis. HLNeo7 control cells (lanes 1) and HL5a1 US11 expressing cells (lanes 2) were heat shock treated for 1 h at 44 °C and allowed recovering for 2 to 8 h before procaspase 8 analysis. Cell extracts were immunobloted with anti-caspase 8 antibody. The p53 and p55 procaspase peptides 8 were quantitated. Note the decreased level of procaspase 8 in control HLNeo7 cells allowed to recover 2 or 4 h after heat shock. (B) Caspase 8 activation analysis. Control HLNeo7 and US11 expressing HL5a1 and HL5e6 cells were treated as described above in (A) and allowed to recover for 2 to 6 h. Activity of caspase 8 was then measured using the fluorescent substrate IETD-AFC as described in Materials and methods. Activation index was determined as the ratio between the activities in extracts of treated cells to that measured in extracts of non-treated cells. Columns represent the mean and error bars represent the standard deviation of three experiments. Note the protective activity of US11 protein that correlates with the inhibition of procaspase 8 cleavage shown above in (A).

heat shock was highly increased in the presence of cycloheximide whereas no processing was observed in US11 expressing cells. Fluorometric analysis of caspase 3 activity was also performed. Results presented in Fig. 5B confirmed that cycloheximide, at the concentration used in this experiment, did not activate caspase 3 in the three cell lines. Interestingly, immediately after heat shock treatment, caspase 3 activity was increased 6.7-fold in control HLNeo7 cells whereas the increase was of only 2.7- and 1.2-fold in the case of HL5a1 and HL5e6 cell lines, respectively. After a 3 h recovery period the increase was up to 8.2-fold in HLNeo7 cells and only 4.4- and 2.7-fold in US11 expressing cells. These results indicate that the interference of US11 with caspase 3 activation process after heat shock was not dependant upon any *de novo* protein synthesis.

US11 also protects against staurosporine induced apoptosis

In order to further analyze the protective function mediated by US11 in HeLa cells, we have analyzed whether this protein could also interfere with the apoptotic process induced by the broad kinase inhibitor staurosporine (Fig. 6). To this aim, HLNeo7 and HL5e6 cells were exposed for 4 h to 0.3 μM of staurosporine. The intensity of the apoptotic process was subsequently estimated by staining the cells with Annexin V/PI (Fig. 6A). Quantitative analysis of the phenomenon revealed that after staurosporine treatment, the percentage of viable HLNeo7 cells (PI and Annexin V negative cells) dropped from 78% to 63% of the total cell population whereas the decrease was far less intense (77% to 72%) in US11 expressing cells. (Fig. 6B) shows a phase-contrast morphological analysis of cells realized after a 3 h treatment with 0.2 μM of staurosporine. HLNeo7 cells presented apoptotic features when compared with US11 expressing cells that had little to no features characteristic of apoptotic cells. Fig. 6C shows the caspases activities determined after staurosporine treatment. Caspases 3 and 8 activities were analyzed after a 4 h of treatment with 0.2 μM staurosporine. A significant 10-fold increase in caspase 3 activation was observed in HLNeo7 cells. In contrast, only a 4.5- and 5.5-fold increase was detected for the HL5a1 and HL5e6 cell lines, respectively. In the case of caspase 8, control HlNeo7 cells showed a 2.5-fold activation level, meanwhile US11 cells did not show any activation after staurosporine treatment. Caspase 9 activation level was determined after a 2.5 h treatment with either a 0.2 or 0.3 μM concentration of staurosporine. In control cells caspase 9 activation was 3.4-fold whereas in HL5a1 and HL5e6 cells it was 2.3 and 1.6 respectively. The

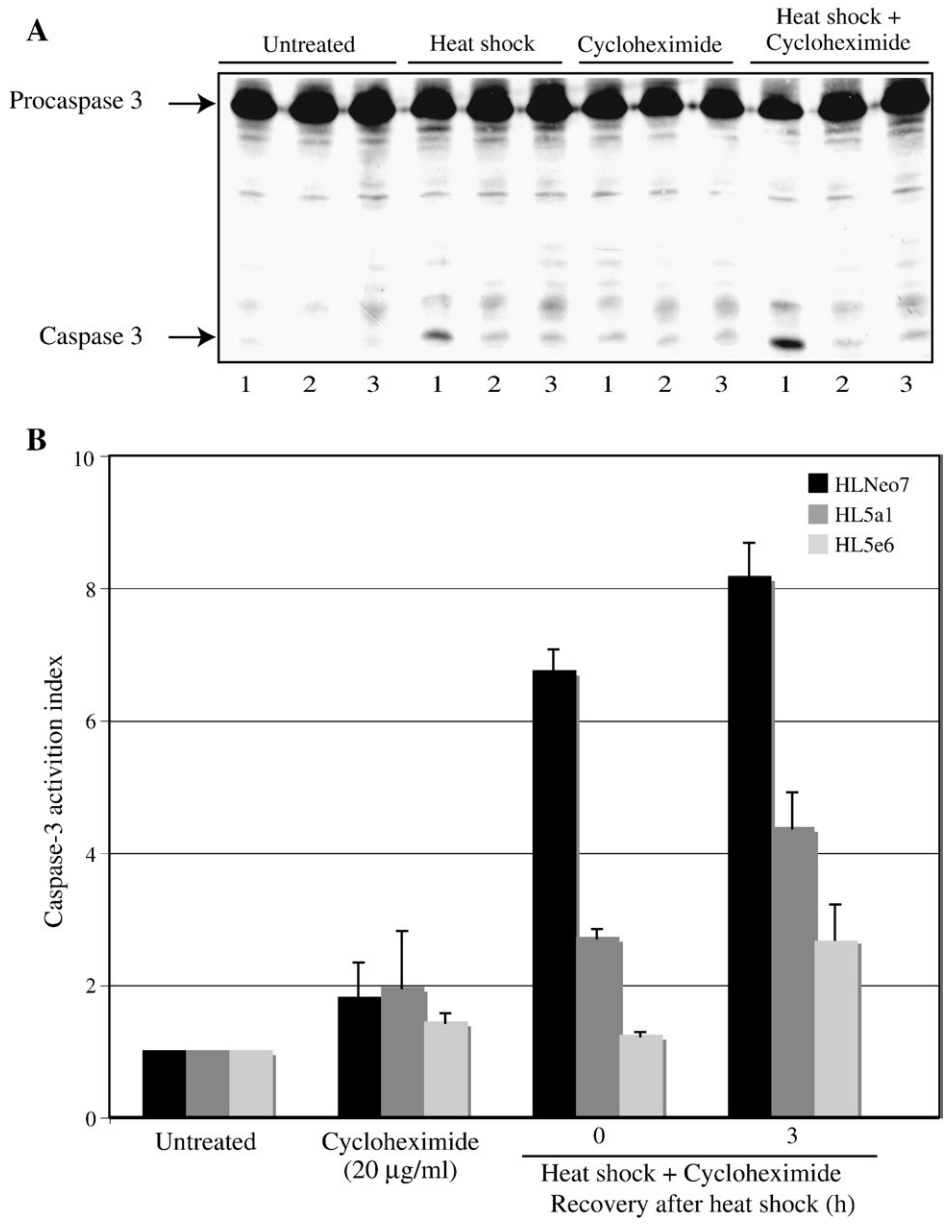


Fig. 5. US11 anti-apoptotic function is independent of Hsp neosynthesis. (A) HLNeo7 control cells (lanes 1) as well as HL5a1 and HL5e6 US11 expressing cells (lanes 2 and 3) were either kept untreated or exposed to a 1 h heat shock at 44 °C followed by a recovery period of 4 h at 37 °C. The experiment was performed in the presence or not of 20 µg/ml of cycloheximide. Cell extracts were immunobloted with anti-caspase 3 antibody. (B) Caspase 3 activation analysis. Control HLNeo7 and US11 expressing HL5a1 and HL5e6 cells were treated with cycloheximide and exposed to heat shock as described above in (A). Activity of DEVD-specific caspases was then measured using the fluorescent substrate DEVD-AFC (see Materials and methods) either immediately after heat shock or after a 3 h recovery period at 37 °C. Activation index was determined as the ratio between activities in extracts of treated cells to that measured in extracts of non-treated cells. Columns represent the mean and error bars represent the standard deviation of three experiments.

differences were obvious after a 0.3 µM treatment as control cells showed a 5.2-fold activation and the Us11 expressing cells a 3.7 and 2.5 activation level. The differences observed were significant as shown by the statistical analysis. These results clearly suggest that the anti-apoptotic activity of US11 is not restricted to heat shock and is also efficient towards staurosporine induced apoptosis.

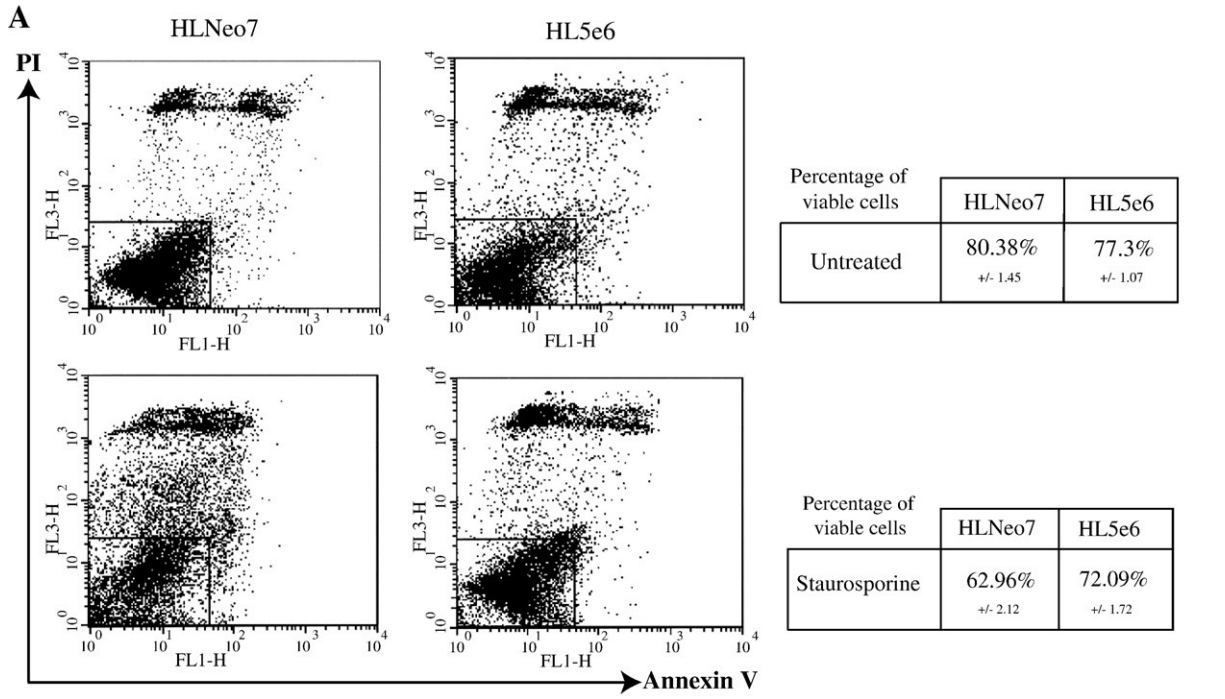
Discussion

Previously, we have demonstrated that the expression of late viral protein US11 of HSV-1 enhanced protein synthesis recovery after heat shock and induced a cellular protection against this stress (Diaz-Latoud et al., 1997). The aim of this work was to determine if US11 was able to protect against heat shock through an anti-apoptotic activity. Consequently, in order to discriminate between the HSV-1 and heat induced apoptosis, we have analyzed the effects mediated by US11 expression alone and not in the context of viral infection. The results presented here show that in heat shock treated HeLa cells, US11 protein highly reduced the number of apoptotic cells, counteracted the dramatic cytochrome *c* efflux from mitochondria and powerfully inhibited the strong activation of the executioner caspase 3.

These results are of major interest in depicting the exact molecular mechanism underlying the heat induced apoptosis. We show a strong activation of caspase 3 and cytochrome *c* release in HeLa cells after heat shock along with minor caspase 8 and caspase 9 activations. This suggests that, in HeLa cells, the FADD pathway is not directly involved to trigger heat induced apoptosis but is probably activated by downstream executioner caspases amplifying the activity of upstream

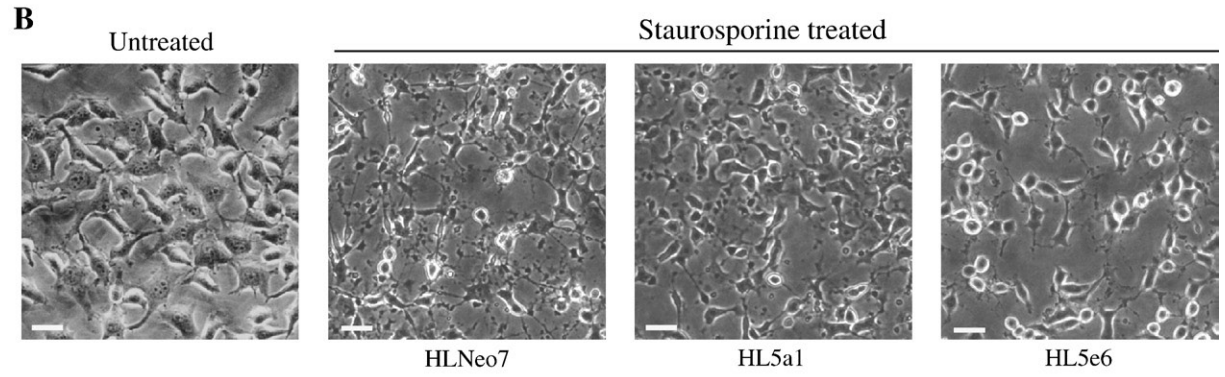
caspases, such as caspase 8 (Wieder et al., 2001). Furthermore the weak caspase 9 activity in HeLa cells after heat shock suggests that caspase 9 activation is not induced by the apoptosome formation after cytochrome *c* release from mitochondria and might result from another activation pathway. These results are strengthened by Milleron and Bratton observations in Jurkat T cells showing that caspase 9 is not essential for activation of caspase 3 or the induction of apoptosis after heat shock (Milleron and Bratton, 2006).

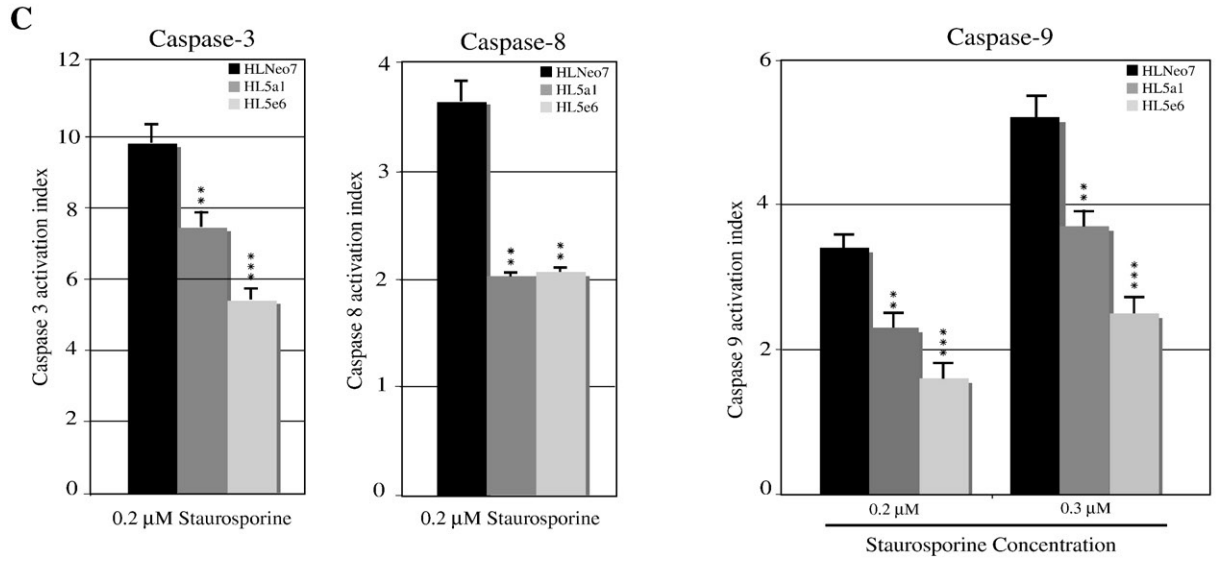
Interestingly, in US11 expressing cells exposed to heat shock, caspase 3 activity is strongly inhibited. In the same conditions MOMP



Percentage of viable cells	HLNeo7	HL5e6
Untreated	80.38% +/- 1.45	77.3% +/- 1.07

Percentage of viable cells	HLNeo7	HL5e6
Staurosporine	62.96% +/- 2.12	72.09% +/- 1.72





is probably highly reduced in the presence of US11 protein since cytochrome *c* efflux is not observed immediately after heat shock but slightly 3 h later and might result from a secondary response. We therefore concluded that this viral protein could interfere either with the heat shock induced mitochondria damage or with upstream signaling pathways activated by the heat shock treatment.

We have already shown that constitutive expression of US11 protein in HeLa cells does not induce any Hsp accumulation (Diaz-Latoud et al., 1997). As Hsp synthesis is an early cell response after heat shock we then tested whether US11 protective effect after heat shock was still observed in conditions where Hsps synthesis was inhibited during and after heat shock by the use of the protein synthesis inhibitor cycloheximide. This resulted in an increased activation of caspase 3 immediately after heat shock, hence confirming the apoptosis protection mediated by Hsps. Whatever in these conditions, the presence of US11 still induced a strong inhibition of caspase 3 activation, a result which indicates that this viral protein generates an anti-apoptotic property that is active in heat shock treated cells and whose function is independent of the presence of newly made Hsps interfering or up-regulating its activity.

Our results provide direct evidence for a causal relationship between the presence of US11 protein and an important delay and reduction in heat shock induced apoptosis in HeLa cells. This points out a mechanism by which HSV-1 could challenge apoptosis in cell submitted to heat stress. Indeed heat stress is one of the most efficient agent to induce reactivation of the latent virus *in vivo* (Roizman, 1990) and is currently used to reactivate the virus in mice latent model (Sawtell and Thompson, 1992).

In order to better characterize this anti-apoptotic activity of US11 protein, we also tested the activity of the viral protein in HeLa cells exposed to the broad apoptotic agent staurosporine. Our results clearly show that US11 expression also strongly decreased apoptosis of the cells and reduced caspase 3, -8 and -9 activations induced by staurosporine.

Altogether these observations demonstrate that US11 protein protects HeLa cells against apoptotic cell death induced by different means, thereby suggesting for this viral protein a novel undescribed function at the level of the apoptotic machinery. US11 could therefore be considered as an anti-apoptotic protein of HSV-1 like already characterized US3, US5, and ICP6 proteins. It has been shown that the accumulation of true late proteins is not required for apoptosis prevention (Aubert et al., 2001). However US11 is brought into the cell by the viral particle and its behavior differs from that of the protein synthesized at the late stage of viral infection (Tan and Katze, 2000). Interestingly it has been recently shown that HSV-1 blocks apoptosis by targeting Bax and precluding cytochrome *c* efflux from mitochondria (Aubert et al., 2007) one might speculate that US11 protein brought into the cell by the virion could be involved in this mechanism. Our results show that US11 preclude early cytochrome *c* efflux from mitochondria during heat shock and it would be of great interest to analyze whether US11 interacts with the pro-apoptotic Bax protein. It would be also of interest to determine if apoptosis is reduced or delayed in US11 expressing HeLa cells during HSV-1 infection.

Whether or not US11 protein could also protect from apoptosis the HSV-1 infected cells submitted to heat shock is an interesting hypothesis that could be tested by analyzing heat shock induced apoptosis in cells infected by virus containing mutations in US11 gene.

Materials and methods

Cells lines

All cells were grown at 37 °C in a humidified atmosphere containing 5% CO_2. HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat inactivated fetal calf serum. Construction and selection of the two clones expressing US11 (HL5a1 and HL5e6) and control cell line (HLNeo7) have already been described (Simonin et al., 1995). For transient expression, exponentially growing HeLa cells were seeded at a density of 1.5×10^6 cells/78 cm^2 one night before transfection with 6.5 µg of pG9 vector according to the Lipofectamine™ reagent procedure (Invitrogen, Cergy Pontoise, France). In the pG9 construct US11 coding sequence is under the control of the cytomegalovirus promoter. The DNA was left on cells for 3 h; cells were then washed once with PBS buffer and further incubated in fresh medium. Twenty-four hours after transfection, cells were submitted to heat shock.

Heat shock was performed by immersing culture dishes into a water bath regulated at 44 °C±0.05 °C.

Reagents

Nonidet P-40, Triton X-100, desoxycholic acid, dithioerythritol, staurosporine, and G418 were from Sigma (St-Louis, MO). Cell culture media and complements were from Invitrogen (Cergy Pontoise, France). The specificity of anti US11-antibody has already been described (Diaz et al., 1993). Anti-caspase 9 antibody was from Stressgen (Victoria, BC, Canada). Anti-cytochrome *c* antibody clone 7H8.2C12, anti-caspase 3 and anti-caspase 8 antibodies were from Pharmingen (San Diego, CA). The primary antibodies were detected with either anti-rabbit or anti-goat immunoglobulins conjugated to horseradish peroxidase, Amersham Corp. (UK). Protease inhibitors, complete cocktail, were from Roche Diagnostics, (Meylan, France).

Measurement of apoptosis

Apoptosis was assessed by determination of the binding of FITC-conjugated Annexin V protein to the phosphatidylserine residues present on the outer leaflet of apoptotic cell membranes as already described (Gonin et al., 1999). Annexin V and propidium iodide (PI) staining were used to differentiate necrotic from apoptotic cells. Samples were analyzed by flow cytometry using a FACS Scan analyzer (Becton Dickinson, Le Pont de Claix, France). Annexin V–FITC and PI related fluorescence was recorded using FL1-H and FL-3H filters respectively.

Measurement of caspase activity

The caspase activity assays were performed with specific fluorometric substrates according to the protocols supplied by the manufacturers. Briefly, after treatment 10^6 cells (caspase 3 and caspase 8 assays) or 2×10^6 (caspase 9 assay) were harvested and subsequently

Fig. 6. US11 protein also reduces apoptosis induced by staurosporine. (A) HLNeo7 control cells or HL5e6 US11 expressing cells were treated for 4 h with 0.3 µM of staurosporine. After treatment, cells were stained with Annexin V and propidium iodide and analyzed as described in Materials and methods. Data are representative of one out of three experiments with comparable results. Lower left square Annexin V and PI negative cells quantification: values represent mean±S.D of three independent experiments. Student's *t* test analysis demonstrated significance $P=0.0005$ of the differences between staurosporine treated HLNeo7 and HL5e6 cell lines. (B) Phase-contrast morphological analysis of cells after staurosporine treatment. HLNeo7 control untreated cells or HLNeo7, HL5a1 and HL5e6 US11 expressing cells treated for 3 h with 0.3 µM of staurosporine. Scale bar=10 µM. (C) HLNeo7 control cells or HL5a1 and HL5e6 US11 expressing cells were treated with staurosporine. Caspase activation was determined as the ratio between the activities in extracts of treated cells to that measured in extracts of non-treated cells. Caspase 3 and caspase 8 activities were analyzed after 4 h of treatment with 0.2 or µM of staurosporine using the fluorescent substrate DEVD-AFC or the fluorescent substrate IETD-AFC respectively. Caspase 9 activity was measured against fluorometric substrate LEHD-AFC, as described in Materials and methods, after 2.5 h of treatment with either 0.2 or 0.3 µM of staurosporine. Columns represent the mean and error bars represent the standard deviation of three experiments. Student's *t* test analysis demonstrated significance ($^{**}P<0.005$ and $^{***}P<0.0001$) of the differences between HLNeo7 and HL5a1 or HLNeo7 and HL5e6 cell lines.

washed twice in ice-cold phosphate-buffered saline (PBS), pH 7.4. Thereafter, cells were spun at 200 ×*g* for 5 min and the dry cell pellets were stored at −80 °C. Caspase 3 and caspase 8 activities were determined by detection of the proteolytic cleavage of DEVD-AFC or IETD-AFC respectively using the ApoAlert fluorescent assay kits (Clontech, Montigny les Bretonneux, France). Determination of caspase 9 activity was performed using the caspase 9 Fluorometric Assay (R&D, Abingdon, UK) which is based on the caspase 9 fluorogenic substrate LEHD-AFC. Quantitation of fluorescence was determined in a Victor Wallach cytofluorometer (EG&G Instruments, Evry, France), excitation was at 400 nm and emission at 505 nm.

Gel electrophoresis and immunoblotting

After heat shock treatment, cells were either immediately scraped or allowed to recover for different time periods at 37 °C before being harvested. Cells were rinsed twice in ice-cold PBS and scraped off the dish. At this point, aliquots were withdrawn for determination of protein concentration. Thereafter, cells were lysed in boiling SDS buffer (62.5 mM Tris–HCl, pH 6.8; 1% SDS; 0.1 M dithioerythritol; 10% glycerol and 0.001% bromophenol blue). Cell lysates were subjected to SDS-PAGE (Laemmli, 1970) and immunoblots were performed as previously described (Diaz-Latoud et al., 2005). The detection of immunoblots was performed with the ECLTM system (Amersham Life Science). Autoradiographs were recorded onto X-Omat LS films (Eastman Kodak Co, Rochester, NY).

Cytochrome c release from mitochondria

The method described by Bossy-Wetzel et al. (1998) was used with some modifications. In brief, about 2×10^6 cells were harvested and subsequently washed twice in ice-cold PBS, pH 7.4. Cells were spun at 200 ×*g* for 5 min and then resuspended in 600 µl of extraction buffer containing protease inhibitors. Cells were incubated for 30 min on ice, then homogenized with a glass dounce and a B pestle (80 strokes). Lysates were spun for 15 min at 14, 000 ×*g*. Thereafter, pellets were directly dissolved in SDS sample buffer while supernatants were diluted 1:1 in 2× sample buffer and boiled for 5 min. Protein analysis was performed in 16.5% SDS-polyacrylamide gels. Gels were then processed for immunoblotting as described before.

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