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ORIGINAL PAPER

Theiler's murine encephalomyelitis virus infection induces a redistribution of heat shock proteins 70 and 90 in BHK-21 cells, and is inhibited by novobiocin and geldanamycin

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Abstract Theiler's murine encephalomyelitis virus (TMEV) is a positive-sense RNA virus belonging to the *Cardiovirus* genus in the family Picornaviridae. In addition to other host cellular factors and pathways, picornaviruses utilise heat shock proteins (Hsps) to facilitate their propagation in cells. This study investigated the localisation of Hsps 70 and 90 in TMEV-infected BHK-21 cells by indirect immunofluorescence and confocal microscopy. The effect of Hsp90 inhibitors novobiocin (Nov) and geldanamycin (GA) on the development of cytopathic effect (CPE) induced by infection was also examined. Hsp90 staining was uniformly distributed in the cytoplasm of uninfected cells but was found concentrated in the perinuclear region during late infection where it overlapped with the signal for non-structural protein 2C within the viral replication complex. Hsp70 redistributed into the vicinity of the viral replication complex during late infection, but its distribution did not overlap with that of 2C. Inhibition of Hsp90 by GA and Nov had a negative effect on virus growth over a 48-h period as indicated by no observable CPE in treated compared to untreated cells. 2C was detected by Western analysis of GA-treated infected cell lysates at doses between 0.01 and 0.125 μM, suggesting that processing of viral precursors was not affected in the presence of this drug. In contrast, 2C was absent in cell lysates of Nov-treated cells at doses above 10 μM, although CPE was evident 48 hpi. This is the first study describing the dynamic behaviour of Hsps 70 and 90 in TMEV-infected cells and to identify Hsp90 as an important host factor in the life cycle of this virus.

Keywords Picornavirus · Hsp90 inhibitors · Cytopathic effect

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Abbreviations

ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
CPE	Cytopathic effect
DAPI	4',6-Diamino-2-phenylindole dihydrochloride
DMEM	Dulbecco's modified Eagle Medium
DMSO	Dimethyl sulphoxide
DTT	Dithiothreitol
EDTA	Ethylene diamine tetra-acetic acid
EMCV	Encephalomyelitis virus
FMDV	Foot-and-mouth disease virus
GA	Geldanamycin
Hop	Hsp70/Hsp90 organising protein
hpi	Hours post-infection
Hsp	Heat shock protein
MOI	Multiplicity of infection
Nov	Novobiocin
PMSF	Phenyl methyl sulphonyl fluoride
PV	Poliovirus
TMEV	Theiler's murine encephalomyelitis virus

Introduction

Picornaviruses are a large family of non-enveloped RNA viruses that includes highly significant human and animal pathogens. Notable examples that are clinically important in humans are poliovirus (PV), coxsakievirus A and B, echovirus 71, human rhinovirus, hepatitis A virus, and parechovirus. Animal pathogens of economic importance are foot-and-mouth disease virus (FMDV) and swine vesicular virus. TMEV and encephalomyelitis virus (EMCV) are type species of the *Cardiovirus* genus, and are pathogens of rodents. Picornaviruses possess a positive-sense RNA genome that is similarly organised in all members of the family. The RNA encodes a single polyprotein which is cleaved co- and post-translationally by viral proteinases into structural (P1) and non-structural (P2–P3) domains. The capsid precursor P1 undergoes further cleavages yielding 60 copies of VP1, VP3 and VP0 which assemble to form the provirion capsid, while P2 and P3 regions yield the replicative polypeptides including 2BC, 2B, 2C, 3A, 3B, 3C and 3D (Fig. 1; for a review see Racaniello 2001). The highly conserved 2C polypeptide has received considerable attention due to its multiple roles in virus replication, and has been shown to target the region of the Golgi apparatus in picornavirus-infected cells (Knox et al. 2005; Jauka et al. 2010).

Members of the *Cardiovirus* genus infect and replicate in cells of the gastrointestinal tract of rodents causing mild or asymptomatic enteric illness, but sometimes, infection can lead to severe systemic disease (Brahic et al. 2005). TMEV isolates differ in virulence: the GDVII and FA strains are highly neurovirulent inducing acute fatal encephalitis in mice, while BeAn and DA strains are less virulent and induce a chronic demyelinating condition. The latter strains have been extensively studied as a model for multiple sclerosis (reviewed by Oleszak et al. 2004). Cardioviruses have traditionally been associated with disease in rodents, but new molecular approaches have led to an expanding list of emerging human pathogens associated with gastrointestinal and neurological diseases within this genus (Jones et al. 2007; Abed and Boivin 2008; Chiu et al. 2008; Drexler et al. 2008; Liang et al. 2008; Blinkova et al. 2009; Zoll et al. 2009). Although their clinical significance remains unclear, human cardioviruses are now the subject of intensive scientific investigation.

Due to the limited coding capacity of their genomes, viruses rely on an array of cellular factors and pathways to complete their replication cycles, and picornaviruses are no exception. Several lines of evidence suggest that molecular chaperones, many of which are also known as Hsps, are notable examples of host factors in this regard. Hsps are a diverse group of proteins classified into different families according to molecular size and play several vital roles in cells including folding of nascent proteins, protein translocation and degradation, prevention of aggregation and cell stress responses (for reviews see Gething and Sambrook 1992; Hartl 1996; Frydman et al. 1994; Frydman 2001; Young et al. 2004). There is substantial evidence to suggest that a wide variety of DNA and RNA viruses differing in host range and replication strategy require the assistance of one or more chaperone pathways to facilitate entry, genome replication and expression, and assembly (for reviews see Sullivan and Pipas 2001; Xiao et al. 2010). In the case of picornaviruses, Hsps 70 and 90 have been found to be essential for the folding and assembly of enteroviruses, as indicated by an association of these chaperones with the capsid P1 precursor (Macejak and Sarnow 1992; Geller et al. 2007).

Hsp90 has received considerable attention for its roles in the regulation of cell growth and differentiation, folding of nascent polypeptides and cell motility associated with cancer metastasis (reviewed in Pratt and Toft 2003). Hsp90 also displays client protein specificity and complexes with Hsp70 and a variety of co-chaperones including immunophilins, p23, Hop and Hsp40 to regulate protein folding and trafficking in cells (Murphy et al. 2001; Picard 2002; Young et al. 2004; Terasawa et al. 2005). Constitutively expressed Hsc70 and stress-inducible Hsp70 play

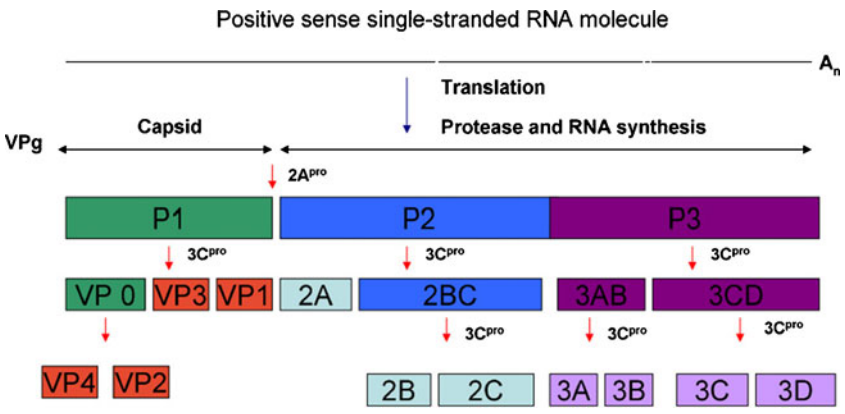

Fig. 1 Picornavirus polyprotein processing. The genome is a positive-sense RNA molecule translated into a polyprotein that is cleaved co- and post-translationally into structural (P1) proteins forming the viral capsid and non-structural P2–P3 replicative polypeptides. 2C is highly conserved and plays multiple roles in virus replication

vital roles in preventing protein aggregation and misfolding in cells, but also function in an ATP-dependent manner to assist the folding of nascent proteins and to guide them from their site of synthesis across organellar membranes (reviewed by Bukau and Horwich 1998; Young et al. 2004).

Pertinent to this study is the growing body of evidence that many viruses have evolved to make use of Hsp90 at different stages of their life cycles including genome replication, (Hu et al. 1997; Hung et al. 2002; Beck and Nassal 2003; Stahl et al. 2007; Smith et al. 2010), viral polymerase localisation and activity (Momose et al. 2002; Burch and Weller 2005; Kampmueller and Miller 2005; Castorena et al. 2007; Connor et al. 2007; Ujino et al. 2009; Smith et al. 2010) and assembly (Macejak and Sarnow 1992; Geller et al. 2007; Naito et al. 2007). The involvement of Hsp90 in the replication cycles of diverse virus species has raised the possibility of developing non-toxic Hsp90 inhibitors as broad-spectrum antiviral agents against a variety of human and animal pathogens.

Although many studies have revealed a role for Hsps 70 and 90 in virus activities, there is little information regarding their distribution in virus-infected cells. In this report, confocal analysis was used to examine the localisation of Hsps 70 and 90 in BHK-21 cells during TMEV infection. In addition, the effect of Hsp90 inhibitors novobiocin and geldanamycin on the development of CPE and production of non-structural protein 2C was monitored over a 48-h period. The results suggest that these chaperones play an important role in the life cycle of TMEV.

Materials and methods

Cells, viruses and infections

BHK-21 cells (kindly provided by M. Ryan, University of St Andrews, UK) were cultured in buffered Dulbecco's modified Eagle Medium (DMEM, Lonza Group Ltd, Basel, Switzerland) supplemented with 10% heat-inactivated foetal calf serum, 100 U penicillin ml^{-1}, 10 mg streptomycin ml^{-1} and 25 μg fungizone ml^{-1} at 37°C with 10% CO_2. TMEV strain GDVII (GenBank accession no: M20562) was used to infect cells in all experiments. Virus stocks were prepared and titred using BHK-21 cells as described previously (Murray et al. 2009). For localisation experiments, cells were infected at a multiplicity of infection (MOI) of 50.

Antibodies and reagents

Rabbit antibodies against non-structural protein 2C were used at a concentration of 1:10,000 for Western analysis, and 1:1,000 for indirect immunofluorescence, and have been described (Jauka et al. 2010). Antibodies recognising Hsp90α and β isoforms (SC-13119) were from Santa Cruz Biotech, Santa Cruz, USA and used at 1:100. Anti-Hsp70/Hsc70 (SMC-104) were from StressMarq, BC, Canada and used at a dilution of 1:500. Species-specific Alexa Fluor 488 and 546-conjugated secondary antibodies (1:500) were from Molecular probes (through Invitrogen, Paisley, UK). Novobiocin (final concentration 250 μM) and geldanamycin (final concentration 0.125 μM) were purchased from Sigma Aldrich, St Louis, USA and resuspended in sterile water and DMSO, respectively.

Nov and GA treatments

Cells were sub-cultured into four six-well plates and grown to 100% confluency. Tenfold dilutions of virus stock (titre: 1×10^8 pfu/ml) were prepared in serum-free DMEM. Spent medium was aspirated and 1 ml of virus dilutions 10^{-5} and 10^{-9} added to duplicate wells of each plate. Two wells of each plate received no virus. Plates were incubated for 1 h with shaking at 37°C to allow virus adsorption. Virus was aspirated and the cells rinsed with sterile phosphate-buffered saline (PBS) before addition of serum-free DMEM containing Nov or GA at final concentrations indicated above. Cells infected in the two untreated plates received either serum-free DMEM alone or serum-free DMEM containing DMSO. Plates were incubated at 37°C and monitored over a 48-h period for the development of CPE. Images were captured 24 and 48 h post-treatment using a Nikon CoolPix 990 light microscope. Controls included virus with no drugs; no virus and no drugs; and no virus and drug only. At the 48-h time point, cells in each plate were collected and lysed, then analysed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and Western analysis using anti-TMEV 2C antibodies as described below. For dose–response analysis, experiments were conducted as above except that Nov was used at final concentrations of 1, 10, 100 and 1,000 μM, and GA at final concentrations of 0.01, 0.1 and 1 μM. At the 24-h time point, images were captured and cells collected and analysed as described above.

Indirect immunofluorescence and confocal microscopy

Cells grown on sterile 13-mm glass coverslips to 100% confluency in six-well plates containing complete medium were washed twice with serum-free DMEM before infection with TMEV (MOI of 50). Control cells were mock-infected with serum-free DMEM. At 6.5 hpi, cells were collected by trypsinisation and washed with PBS before fixation with 4% paraformaldehyde for 20 min at room temperature (RT). For staining, cells were permeabilised in

PB (10% sucrose, 1% Triton X-100 in PBS), blocked in PB containing 2% BSA for 30 min at RT and incubated with primary antibodies for 1 h with shaking. Cells were washed with PBS containing 0.1% Tween-20, and incubated with species-specific Alexa Fluor 488-conjugated and/or Alexa Fluor 546-conjugated secondary antibodies for 30 min, followed by three washes. To stain the nucleus, 4',6-diamino-2-phenylindole dihydrochloride (DAPI, Sigma, St Louis, USA) was added at a concentration of 0.8 μg/ml in the second wash step. Cells were mounted using Dako fluorescence mounting medium (Dako Inc, CA, USA) and stored at 4°C. For time course experiments to monitor the distribution of Hsp90, cells were fixed at 4, 6 and 8 h post-infection and stained as above. Cells were visualised using an inverted LSM 510-Meta confocal laser scanning microscope (Carl Zeiss, Germany) using the ×63 oil immersion objective lens. The helium/neon and argon lasers at wavelengths 405, 488 and 543 nm were used to excite DAPI, Alexa-fluor 488 and Alexa-fluor 546, respectively. Images were analysed and processed using the Axiovision LE/SE freeware software (Carl Zeiss, Germany).

Preparation of infected cell lysate for Western analysis

For Western analysis, cells were collected by centrifugation at 1,000×*g* and lysed in 100 μl lysis buffer [50 mM Tris–HCl, (pH 7.5), 150 mM NaCl, 2% Triton X, 1 mM PMSF, 1 mM DTT, 1× EDTA-free protease inhibitor cocktail (Roche, Mannheim, Germany)] on ice for 30 min with vigorous vortexing every 5 min. Proteins were denatured and resolved by 12% SDS–PAGE before transfer onto nitrocellulose membrane for Western analysis using anti-TMEV 2C antibodies. The BM Chemiluminescence Western Blotting kit (Roche, Mannheim, Germany) was used to detect viral protein according to manufacturers' instructions.

Results

Hsp90 redistributes into the perinuclear region during TMEV infection

To examine the effect of virus infection on Hsp90 localisation, cells were infected with TMEV, fixed at 6.5 hpi, and co-stained with anti-Hsp90 and anti-TMEV 2C antibodies (Fig. 2). 2C is a non-structural protein with multiple roles in virus replication. We have recently shown that the protein is present in TMEV-infected cells at 4 hpi and is targeted to the Golgi region where it participates in the formation of the viral replication complex (Jauka et al. 2010; Murray et al. 2009). In the majority of uninfected cells, the signal for Hsp90 was distributed uniformly in the cytoplasm with faint nuclear staining (Fig. 2a). In most of the TMEV-infected cells examined (Fig. 2b), there was still significant cytoplasmic staining, but also redistribution of the Hsp90 signal into the perinuclear region where it

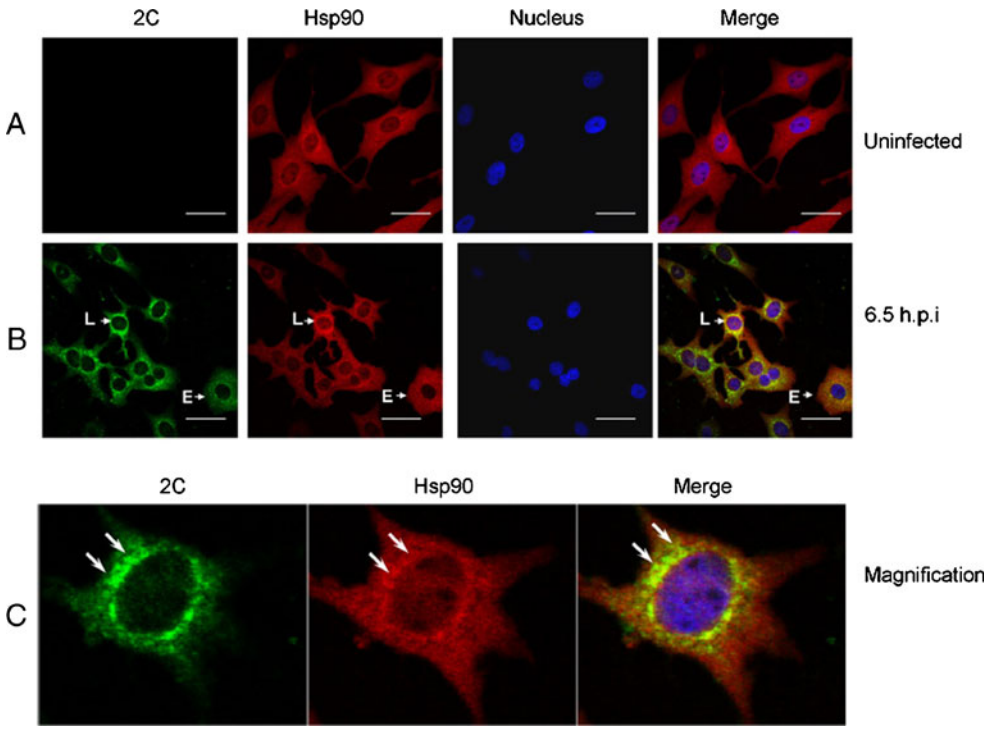

Fig. 2 Hsp90 distribution in uninfected cells and at 6.5 hpi. Cells were infected with TMEV, fixed with paraformaldehyde at 0 hpi (**a**) or 6.5 hpi (**b**, **c**) and co-stained with antibodies against 2C and Hsp90. Primary antibodies were detected with species-specific Alexa Fluor 488- or Alexa Fluor 546-conjugated secondary antibodies. In **b**, *letters E* and *L* indicate cells in early and later stages of infection, respectively (see text for explanation). In the *magnified image*, note the concentration of Hsp90 in the perinuclear region where it overlaps with the 2C signal (*arrows*; **c**). *Scale bar*=20 μm

overlapped with 2C. This observation is highlighted in the panel by indicating a cell in the early stage of infection (E) and a cell in a later stage of infection (L). In magnified images of infected cells (Fig. 2c, arrows), this redistribution was prominent, and there was also evidence of Hsp90 staining in the nucleus. To examine the distribution of Hsp90 over time, infected cells were fixed at 4, 6 and 8 hpi and stained as above. As shown in Fig. 3, the redistribution of Hsp90 is clearly time-dependent. At 4 hpi, when there was faint 2C staining representative of early infection (panel a), Hsp90 was localised uniformly in the cytoplasm with negligible nuclear staining. At 6 hpi, when 2C was prominent in the perinuclear region, the Hsp90 signal was still distributed in the cytoplasm but was also concentrated in the perinuclear region in most of the cells where it overlapped with 2C (panel b). At the 8-h time point, the viral replication complex was clearly visible, and some of the Hsp90 signal was found overlapping with 2C and was also present in the nucleus (panel c).

Hsp70 redistributes to the vicinity of the viral replication complex in TMEV-infected cells

The next experiment investigated the distribution of Hsp70 at 6.5 hpi. Cells were infected or not with TMEV, fixed and co-stained with anti-Hsp/Hsc70 and anti-TMEV 2C antibodies. Figure 4a shows the signal for Hsp70 in punctate structures which are distributed throughout the cytoplasm of uninfected cells. At 6.5 hpi, 2C staining was clearly visible in some of the cells in a large juxta-nuclear structure representing the viral replication complex (panel b). The Hsp70 signal was still punctate but concentrated in the vicinity of the replication complex in most of the cells examined. This observation is highlighted in panel b by indicating a cell in the early stage of infection (E) and a cell in a later stage of infection (L). In contrast to Hsp90, there was no significant overlap between the Hsp70 and 2C signals and little evidence of Hsp70 nuclear staining (Fig. 4c).

Hsp90 inhibitors Nov and GA inhibit the onset of CPE in TMEV-infected cells

To investigate the effect of Hsp90 inhibitors on the development of CPE, cells grown to confluency in six-well plates were infected or mock-infected with TMEV in the presence or absence of 250 μM Nov or 0.125 μM GA and examined at 24 and 48 hpi. CPE in these experiments was defined as disruption of the confluent monolayer, detachment of opaque cells from the growth surface, and formation of cell aggregates. Figure 5a depicts light microscopy images of cells subjected to various treatments as indicated, captured at 48 hpi. CPE was not well-developed at the 24-h time point in infected cells (data not shown). At 48 hpi, CPE was clearly observed in TMEV-infected but not mock-infected cells in the absence of drugs (Fig. 5a, upper panels). In infected cells treated with Nov or GA (Fig. 5a, middle and lower panels respectively), rounding up and detachment of cells occurred at this time point, but typical CPE indicative of virus infection was not evident, and cells were translucent rather than opaque. Interestingly, cell detachment was more severe in Nov- compared to GA-treated cells at 48 hpi (compare middle and lower panels of Fig. 5a). To test for the presence

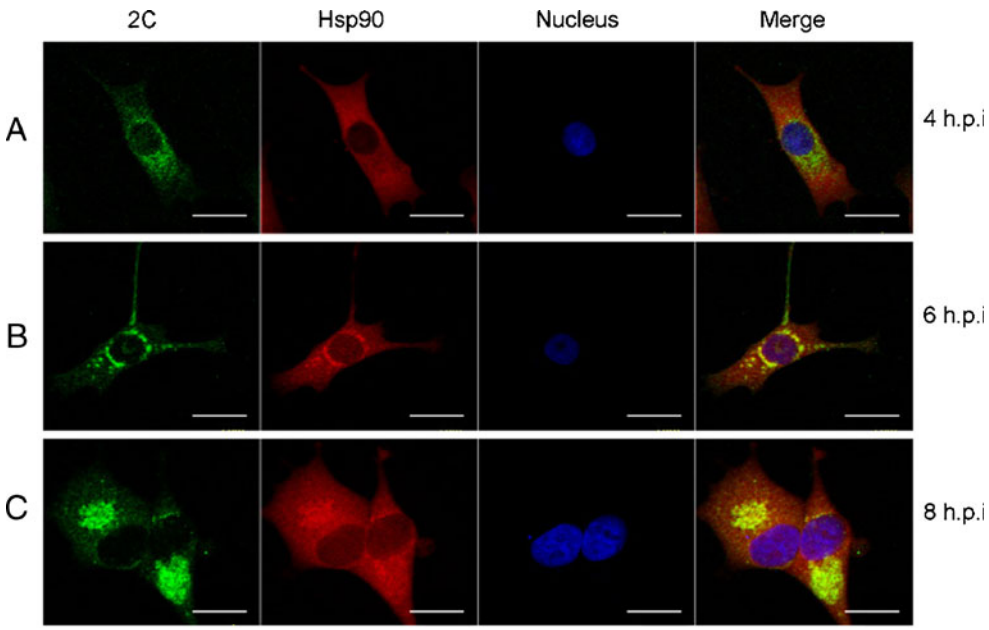

Fig. 3 Hsp90 distribution in TMEV-infected cells is dependent on stage of infection. Cells were infected with TMEV, fixed at 4, 6 and 8 hpi with paraformaldehyde and co-stained with antibodies against 2C and Hsp90. Primary antibodies were detected with species-specific Alexa Fluor 488- or Alexa Fluor 546-conjugated secondary antibodies. The Hsp90 signal appears to concentrate in the perinuclear region during infection (compare *top* and *middle panels*). During late infection, Hsp90 is also clearly present in the nucleus (*lower panels*). *Scale bar*=20 μm

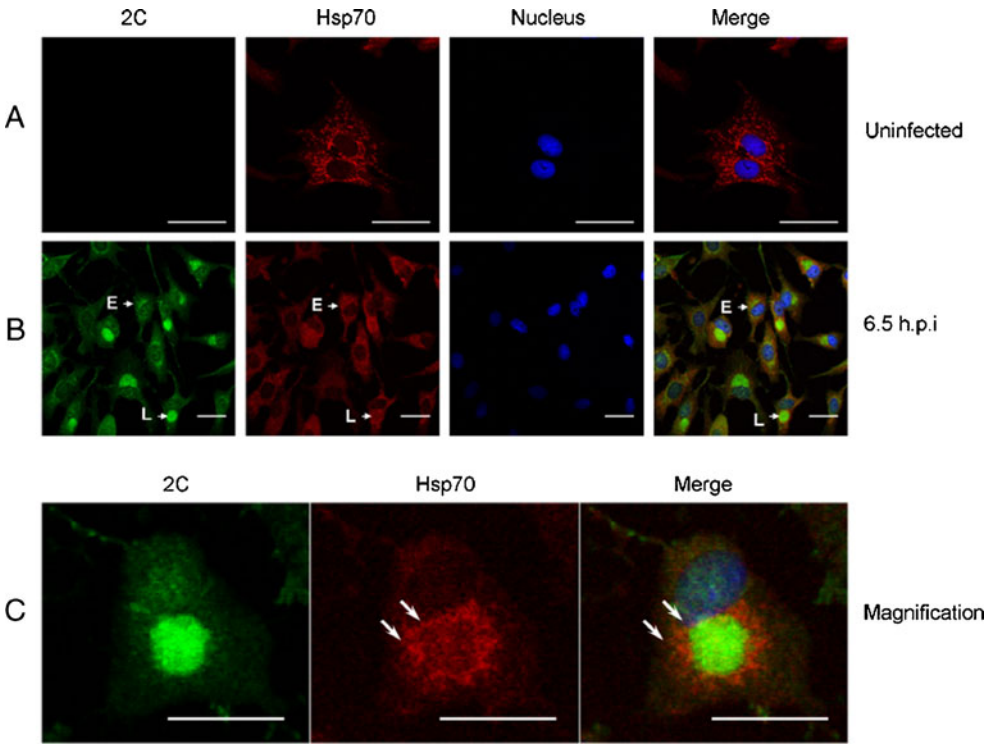

Fig. 4 Hsp70 redistributes to the vicinity of the replication complex in TMEV-infected cells. Cells were infected with TMEV, fixed with paraformaldehyde at 0 hpi (**a**) or 6.5 hpi (**b**, **c**), and co-stained with antibodies against 2C and Hsp70. Primary antibodies were detected with species-specific Alexa Fluor 488- or Alexa Fluor 546-conjugated secondary antibodies. In **b**, *letters E* and *L* indicate cells in early and later stages of infection, respectively (see text for explanation). In the magnified images, note the concentration of Hsp70 in the vicinity of the viral replication complex containing the 2C signal (*arrows*; **c**). *Scale bar*=20 μm

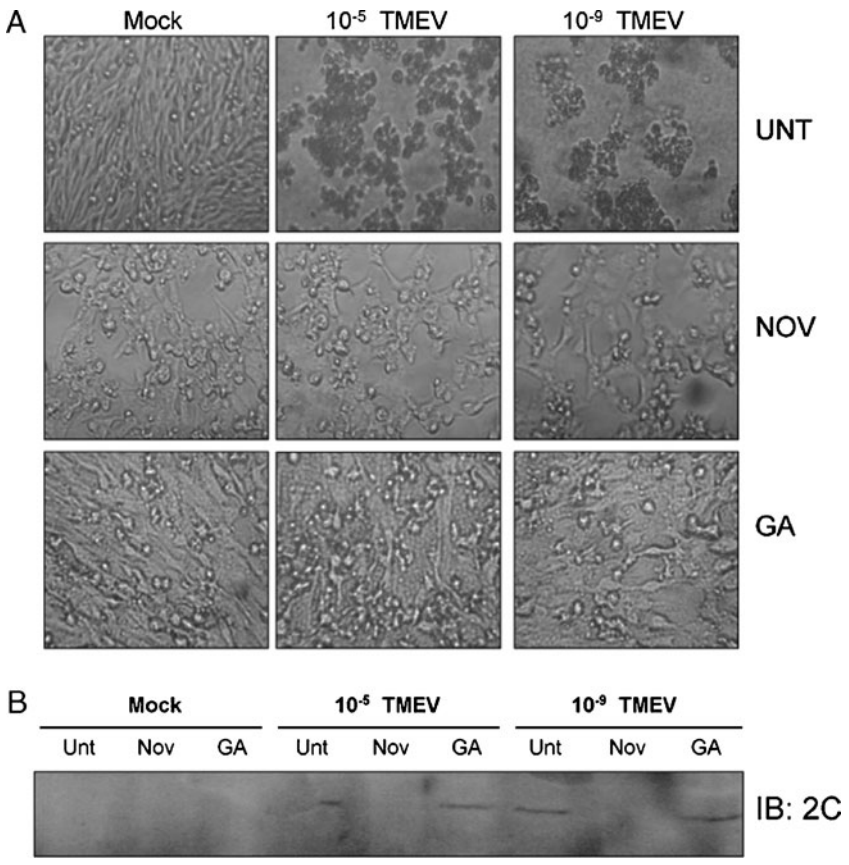

Fig. 5 Effect of Hsp90 inhibitors on TMEV infection by indirect immunofluorescence and Western analysis at 48 hpi. **a** Cells were infected or mock-infected with TMEV, and monitored for the development of CPE in the absence (*upper panels*) or presence (*lower panels*) of 250 μM Nov or 0.125 μM GA. Images were captured using a Nikon CoolPix 990 light microscope at ×2 magnification. **b** Immunoblotting (IB) of mock-infected and infected cell lysates in the presence or absence of either Nov or GA using anti-TMEV 2C antibodies. Note the absence of the 2C signal in the Nov-treated sample

of virus, cells were collected from each well, lysed at the 48-h time point and subjected to SDS–PAGE and Western analysis using anti-TMEV 2C antibodies (Fig. 5b). As expected, 2C was not detected in mock-infected lysates either in the absence or presence of drugs. Also expected was a clear 2C signal in infected, untreated lysates. Interestingly, a prominent signal for 2C was present in infected lysates treated with GA, but not in Nov-treated samples at the dose used.

Dose response experiments with Nov and GA

To further investigate the effects of Hsp90 inhibitors on virus infection, dose–response experiments were performed using 10^{-5} and 10^{-9} TMEV-infected cells (Fig. 6a, b). Rounding up and detachment of cells were observed in GA- and Nov-treated cells at the 48-h time period in previous experiments (see Fig. 5a), an effect likely to be exacerbated by higher doses of these drugs. For this reason, cells were imaged and collected for lysis and Western analysis at 24 hpi. The first signs of CPE were evident in TMEV-infected cells, particularly at the 10^{-5} virus dilution as seen by opaque, detached cells (Fig. 6a, arrows in top panels). CPE was not observed in infected cells treated with 0.01 or 0.1 μM GA (Fig. 6a, middle panels). At the 1 μM GA dose, CPE was not also observed, although rounding up and detachment of cells occurred at this time point even in mock-infected cells (Fig. 6a, arrows in lower panels). When lysates were probed with anti-TMEV 2C antibodies, 2C protein was not present in either mock-infected or virus-infected cells treated with 1 μM GA. A signal for 2C was detected in infected cells treated with 0.01 and 0.1 μM GA at the lower TMEV dilution of 10^{-5} only (Fig. 6a).

Similar experiments were conducted using various doses of Nov (Fig. 6b). Obvious signs of CPE were evident in some cells at the early time point of 24 hpi. Nov doses of 100 and 1,000 μM induced morphological changes in cells that did not resemble CPE (Fig. 6b, lower panels). Probing of infected cell lysates with anti-TMEV 2C antibodies revealed signals for the protein in 10^{-5} (but not 10^{-9}) TMEV-infected cells at Nov doses below 100 μM (Fig. 6b).

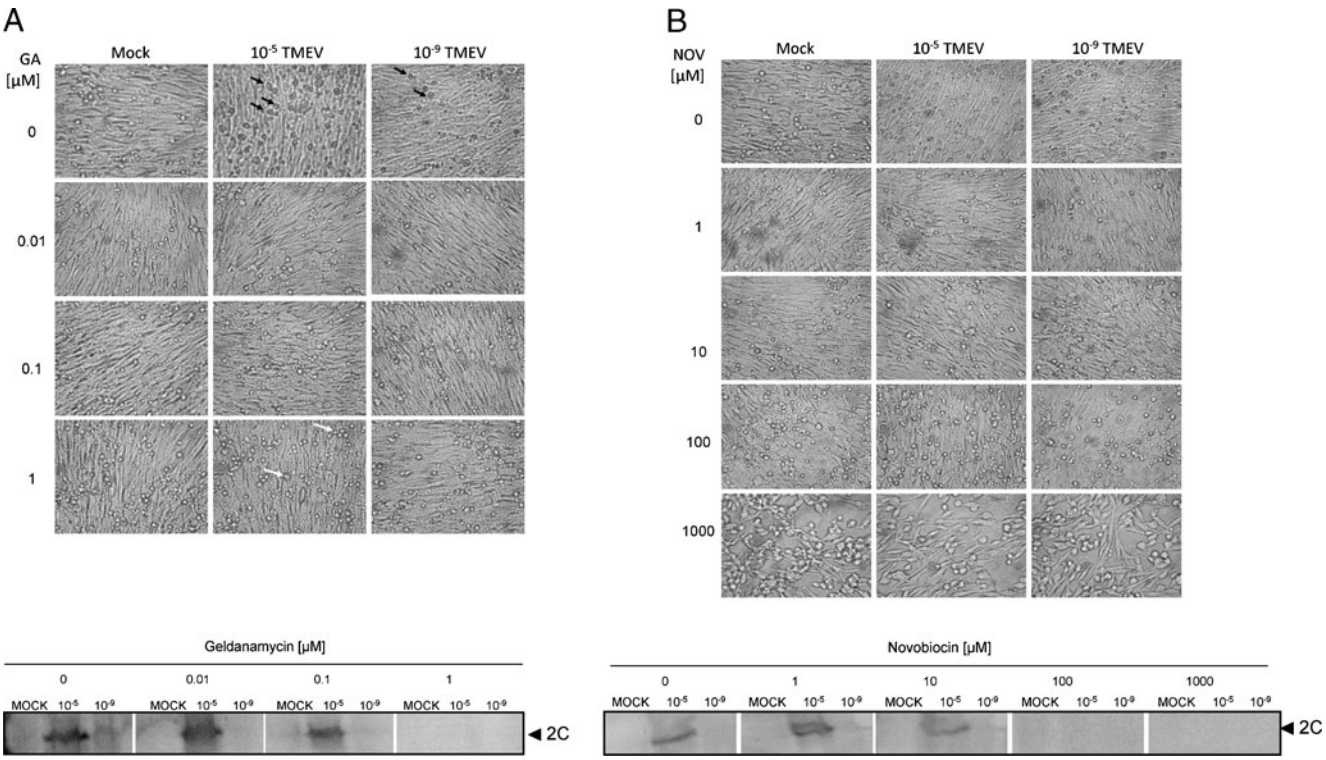

Fig. 6 Effect of various doses of Hsp90 inhibitors on TMEV infection by indirect immunofluorescence and Western analysis at 48 hpi. Cells were infected or mock-infected with TMEV, and monitored for the development of CPE in the absence (*upper panels*) or presence (*middle* and *lower panels*) of 0.01, 0.1 and 1 μM GA (**a**) or 1, 10, 100 and 1000 μM Nov (**b**). Images were captured using a Nikon CoolPix 990 light microscope at ×2 magnification. Immunoblotting of cell lysates using anti-TMEV 2C antibodies in the presence or absence of GA and Nov is shown below the image data in **a** and **b**, respectively. In Fig. 6a, note the opaque, rounded cells undergoing CPE (*black arrows*, *upper panels*) and translucent, rounded appearance of cells treated with GA (*white arrows*, *lower panel*)

Discussion

The experiments described herein indicate through indirect immunofluorescence, observation of CPE and Western analysis that Hsps 70 and 90 are host factors that play a role in productive TMEV infection. This is not surprising given the wealth of information reporting an involvement of these chaperone families in the life cycle of not only picornaviruses, but also a wide variety of RNA viruses (see for example, Macejak and Sarnow 1992; Vasconcelos et al. 1998; Guerrero et al. 2002; Momose et al. 2002; Tomita et al. 2003; Kampmueller and Miller 2005; Kumar and Mitra 2005; Geller et al. 2007; Connor et al. 2007; Naito et al. 2007; Smith et al. 2010). To our knowledge, however, this is the first study reporting the dynamic behaviour of Hsps 70 and 90 in picornavirus-infected cells. Firstly, immunofluorescence analysis of infected cells demonstrated a redistribution of Hsp90 into the perinuclear region and nucleus of TMEV-infected cells in a manner that was clearly dependent on stage of infection. This observation was confirmed by imaging over a hundred 2C-positive cells at each time point post-infection (data not shown). It is important to note that the antibodies used for detection in these experiments recognise both alpha and beta Hsp90 isoforms. It will be interesting, therefore, to explore the possibility that one or other Hsp90 is responding uniquely to the viral infection by performing similar experiments with isoform-specific antibodies.

In contrast to Hsp90, rather than sorting to the nucleus, Hsp70 was found concentrated around the 2C-containing viral replication complex during late infection. The dynamic intracellular reorganisation of Hsp90/70 under conditions of cellular stress was reported years ago and is well documented. Both chaperones, for example, are sorted to the nucleus upon heat stress and reappear in the cytoplasm during cell recovery where they possibly participate in protection and repair of the cellular organelle structure following stress events (Pelham 1984; Collier and Schlesinger 1986; reviewed by Snoeckx et al. 2001). Although the biological significance of the observations in this study are unclear at this stage, Hsp70 has been reported to form a stable association with the P1 capsid precursor in PV- and coxsakievirus B1-infected cells, and is proposed to play a role in virus assembly (Macejak and Sarnow 1992). More recently, the picornavirus P1 precursor was identified as a folding substrate of Hsp90, and a model proposed whereby the P1 precursor was bound by Hsp90 and folded with the assistance of co-factors such as Hsp70 and p23 into a conformation conducive for maturational cleavage by the viral 3C protease (Geller et al. 2007). An interesting question arising from the present study is why Hsp70 is not translocated into the nucleus as is observed during cell stress situations. Perhaps, as suggested by Macejak and Sarnow (1992), Hsp70 is sequestered by nascent P1 in the cytoplasm where it not only facilitates folding of the capsid precursor into a particular conformation required for productive cleavage but also mimics its natural role in the cell by accompanying nascent proteins from the site of synthesis to membranes (Bukau and Horwich 1998), in this case those of the replication complex.

Confocal analysis of TMEV-infected cells revealed that although a significant proportion of the Hsp90 signal remained in the cytoplasm, it became concentrated in the region of the viral replication complex over time. The biological significance of this localisation pattern is unclear at this stage, but it suggests that Hsp90 is required in the late stages of TMEV infection. Hsp90 has been identified as an essential folding factor for the capsid precursors of three major picornavirus pathogens, and given the highly conserved nature of this virus family, it is likely that a requirement for Hsp90 during virion assembly extends to other genera. Movement of Hsp90 into the nucleus of infected cells during infection was also observed in this study. Geller and co-workers (Geller et al. 2007) proposed a model whereby cleavage of PV P1 precursors into mature capsid proteins is accompanied by release of Hsp90 into the cytoplasm. It is tempting to speculate, therefore, that an involvement of Hsp90 in TMEV infection, if it exists, is transient and dynamic permitting much of the protein to perform "normal" stress-related functions in the cell. Additionally, Hsp90 is ubiquitous and abundant in cells, and it is possible that only a proportion of the total pool is recruited to participate in viral activities.

To further understand the role of Hsp90 in TMEV infection, the development of CPE in the presence and absence of well-known Hsp90 inhibitors was examined. In the absence of drugs, CPE was well developed in cells at 48 hpi but was less evident during early infection at the 24-h time point, especially at the 10^{-9} virus dilution. At doses of 0.125 and 250 μM, respectively, GA and Nov prevented the onset of CPE in TMEV-infected cells over a 48-h period. GA, a naturally occurring antibiotic, binds to a hydrophobic pocket within the N-terminal ATP-binding domain of Hsp90 locking it into an ADP-dependent configuration unable to bind co-chaperone p23 (Schneider et al. 1996; Grenert et al. 1997; Stebbins et al. 1997; Sullivan et al. 1997). Consequently, progression through the ATP-driven maturation and folding reaction is inhibited, leading to accumulation of intermediate Hsp90 complexes and proteasome-mediated degradation of substrates. Our observations are consistent with studies showing that GA impairs the replication and assembly of RNA viruses by inhibiting Hsp90 function (Geller et al. 2007; Chase et al. 2008; Connor et al. 2007; Kampmueller and Miller 2005; Smith et al. 2010). In the case of picornaviruses, abrogation of the p23–P1 interaction by GA was proposed to block progression through the Hsp90 folding pathway and

decrease the production of mature virus capsids by targeting P1 for degradation (Geller et al. 2007). The presence of a prominent signal for 2C detected by Western analysis of TMEV-infected cells treated with 0.125 μM GA indicated that early, post-entry stages of TMEV-infection were not affected. This is consistent with the finding that GA treatment did not affect translation or replication of a poliovirus replicon, or the processing and maturation of P2- and P3-derived polypeptides in poliovirus-infected cells (Geller et al. 2007). Based on these observations, the production of 2C was monitored in cells treated with varying doses of GA. A post-infection time point of 24 h was chosen in order to collect cells for 2C analysis prior to cell detachment and possible lysis due to toxic effects of the drug. At this early time point, CPE was not well developed although the first signs were apparent in 10^{-5} TMEV-infected cells. The presence of a 2C signal in GA-treated cells at doses of 0.01 and 0.1 μM at the 10^{-5} TMEV dilution is consistent with previous experiments and indicates, once again, that this drug does not affect early post-entry events. Interestingly, no signal for 2C was observed in infected lysates treated with 1 μM GA. The biological significance of this result requires further investigation but may relate to toxicity effects at this dose, which is well above that used in similar experiments (Geller et al. 2007).

Although the anti-viral effect of Nov was reported years ago (Franke and Margolin 1981; Landini and Baldassarri 1982; Sumiyoshi et al. 1983), to date, no studies have described its effect on picornavirus infection. Novobiocin, a member of the coumarin group of antibiotics, exhibits potent activity against Gram-positive bacteria by inhibiting ATP hydrolysis (Reece and Maxwell 1991). Studies have revealed that Nov competes with ATP for binding to a region on Hsp90 overlapping the carboxyl-terminal dimerisation domain, leading to disruption of Hsp90 multi-chaperone complexes and subsequent client protein degradation (Marcu et al. 2000a, b; Yun et al. 2004; reviewed by Donnelly and Blagg 2008). As observed for GA, cells infected with TMEV monitored over a 48-h period in the presence of Nov did not display signs of virus-induced CPE, indicating that it has an inhibitory effect on virus replication and/or assembly. Interesting, however, was the observation that in three independent experiments, 2C was not detected by Western analysis of Nov-treated infected cell lysates when the drug was used at a concentration of 250 μM. This was also the case in dose–response experiments where Nov was present at concentrations of 100 and 1,000 μM. These results contrasted with those obtained for GA and may be a consequence of the different mechanisms of Hsp90 inhibition involved. GA, for example, causes the accumulation of intermediate Hsp90 complexes by preventing formation of the "closed clamp" Hsp90 conformation (Grenert et al. 1997). Nov-mediated inhibition, on the other hand, is thought to promote disassembly of late Hsp90 multi-protein complexes leading to client protein release and degradation (Yun et al. 2004). It should be noted, however, that treatment of cells with Nov, even in the absence of virus, led to detachment from the growth surface at the 48-h time point at a dose of 250 μM, and at 24 hpi at doses of 100 and 1,000 μM. The Nov doses chosen for these provisional experiments were based on previous studies of this kind where a similar range of concentrations was used in various cells types (Landini and Baldassarri 1982; Sumiyoshi et al. 1983; Lührmann et al. 1998; Singh et al. 2005). The results presented here indicate that BHK-21 cells may be more susceptible to the effects of Nov than are other cell lines, and therefore not able to support translation and processing of viral non-structural proteins at the doses used. A second explanation for the apparent sensitivity of the cells to Nov in the presence and absence of virus infection, and for the absence of a 2C signal, could be related to the anti-topoisomerase activity of novobiocin which is well documented (Reece and Maxwell 1991; Robinson et al. 1993; reviewed by Larsen et al. 2003). Perhaps, this property of Nov influences cellular processes which are required for production and processing of viral non-structural proteins including 2C. Investigating this possibility was beyond the scope of this study, but it will be interesting in future studies to use alternative anti-topoisomerases in order to examine the effects of novobiocin that are not related to Hsp90 inhibition. Finally, although there is no evidence that 2C associates with Hsp90 in infected cells, it is tempting to speculate that Nov-induced destabilisation of late Hsp90 multi-protein complexes causes its degradation in infected cells. In addition to its canonical roles in virus replication and membrane proliferation, for instance, the multi-functional 2C polypeptide is also implicated in encapsidation of viral RNA during the final stages of virion assembly (Vance et al. 1997). Future studies will address these possibilities by conducting dose response experiments in Nov-treated cells, and monitoring the presence of 2C at various stages post-infection.

The mechanism of action of Hsps 70 and 90 during TMEV infection remains to be determined. However, the confocal data in this report indicate a redistribution of these chaperones in a manner which, in the case of Hsp90, is clearly dependent on stage of infection. One interesting question arising from these experiments is whether pre-existing or newly synthesised Hsps are redistributed as a result of viral infection. The focus of future studies will involve monitoring levels of Hsp90 and Hsp70 expression in infected cells at various time points post-infection, and correlating these with changes in distribution during the course of infection. A second interesting question is

whether the signals for Hsp90 and Hsp70 co-localise upon redistribution. Both proteins, for example, have very different distribution patterns in uninfected cells, yet they concentrate in the vicinity of the 2C-containing replication complex during later infection. It will be interesting to investigate the possibility of co-localisation within or at the periphery of the replication complex which would suggest that both proteins are acting synergistically to enhance viral activities.

Western analysis of infected cells lysates showed the presence of 2C in GA-treated cells supporting the model proposed by Geller and co-workers (Geller et al. 2007), and suggesting a role for Hsp90 during late stages of TMEV infection. It is clear from the present experiments that Nov (at the doses used) inhibits the development of CPE in infected cells. However, the absence of a signal for 2C in Nov-treated cell lysates at doses of 100 μM and above is interesting and may be indicative of an additional role for Hsp90 in virus activities. Future experiments will build on these data by investigating the effect of Nov on viral protein production and localisation by immunofluorescence analysis of infected cells.

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