Nuclear translocation of the phosphoprotein Hop (Hsp70/Hsp90 organizing protein) occurs under heat shock, and its proposed nuclear localization signal is involved in Hsp90 binding

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Abstract

The Hsp70–Hsp90 complex is implicated in the folding and regulation of numerous signaling proteins, and Hop, the *H*sp70–Hsp90 *O*rganizing *P*rotein, facilitates the association of this multichaperone machinery. Phosphatase treatment of mouse cell extracts reduced the number of Hop isoforms compared to untreated extracts, providing the first direct evidence that Hop was phosphorylated in vivo. Furthermore, surface plasmon resonance (SPR) spectroscopy showed that a cdc2 kinase phosphorylation mimic of Hop had reduced affinity for Hsp90 binding. Hop was predominantly cytoplasmic, but translocated to the nucleus in response to heat shock. A putative bipartite nuclear localization signal (NLS) has been identified within the Hsp90-binding domain of Hop. Although substitution of residues within the major arm of this proposed NLS abolished Hop–Hsp90 interaction as determined by SPR, this was not sufficient to prevent the nuclear accumulation of Hop under leptomycin-B treatment and heat shock conditions. These results showed for the first time that the subcellular localization of Hop was stress regulated and that the major arm of the putative NLS was not directly important for nuclear translocation but was critical for Hop–Hsp90 association in vitro. We propose a model in which the association of Hop with Hsp90 and the phosphorylated status of Hop both play a role in the mechanism of nucleo-cytoplasmic shuttling of Hop.

1. Introduction

The *H*sp70–Hsp90 *O*rganizing *P*rotein, Hop, was first identified in yeast during a genetic screen for proteins involved in the heat shock response [1]. Hop has insignificant chaperoning abilities [2] and upon heat shock (HS) and viral transformation, no corresponding change in the steady state levels of this protein was detected, although an increase in mRNA levels of Hop in mammalian cells was observed [3] and [4]. Hop acts as a scaffolding protein, mediating the interaction of the molecular chaperones Hsp70 and Hsp90 through specific tetratricopeptide-repeat (TPR)-rich binding domains [5] and [6] to form the Hsp70–Hop–Hsp90 chaperone heterocomplex. These

TPR domains characterize the various homologues of Hop, found in humans [3], mice [7], rats [8], insects [9], plants [10], parasites [11] and viruses [12]. Hsp70 binding is mediated primarily through the N-terminal TPR domain of Hop (TPR1) [13] while a central TPR motif-containing region (TPR2A) is known to be essential for Hsp90 binding [14] and [15]. The TPR-acceptor site on both Hsp70 and Hsp90 comprises an EEVD motif at the C-terminus [15] and [16].

In the yeast system, it was recently shown that the third TPR domain, TPR2B is also directly involved in Hop's interactions with Hsp70 and Hsp90, and that an isolated TPR2A domain is unable to interact with Hsp90 unless combined with TPR2B [17]. Deletion of a specific amino acid in the TPR2B domain of yeast Hop (A438) showed the disruption of in vivo interaction with Hsp90 [18]. The possibility of a network of interactions between Hop and the chaperones Hsp70 and Hsp90, involving domains other than the TPRs, has been suggested, which support its functionality as a scaffolding protein [15] and [19].

Early studies reported the presence of human Hop in the Golgi apparatus and small vesicles of normal cells, and in the nucleolus of SV40-transformed cells [3]. Immunoprecipitation studies using membranous fractions from mouse brain showed that a population of Hop is localized at the cell surface, where it interacts with Prp^c (a protein whose expression is crucial to the propagation of neurological disease, in particular, Prion disease) both in vitro and in vivo. It has been proposed that Hop is taken into the plasma membrane as part of a Prp^c –protein complex [20]. Alternatively, Hop may be secreted by a pathway that is distinct from the classical route of membrane translocation through the ER and Golgi apparatus [21] since a transmembrane domain or signal peptide for membrane transport is not found within the amino acid sequence of Hop [4] and [20].

An investigation into the subcellular localization of Hop in mouse cells showed a predominantly cytoplasmic localization under normal growth conditions [4]. Leptomycin-B treatment (nuclear export inhibition) of mouse fibroblast cells, resulted in a predominantly nuclear accumulation of Hop, suggesting that the protein shuttled between the nucleus and the cytoplasm, with the export of Hop from the nucleus occurring at a faster rate than its import [22]. Moreover, Longshaw et al. identified a potential bipartite nuclear localization signal (NLS), which when fused to enhanced green fluorescent protein (EGFP), resulted in the localization of EGFP to the nucleus [22], providing evidence that this NLS was indeed functional on its own. This putative NLS comprised of a short lysine arm (K222–K223), a 13-amino acid spacer region, followed by a major lysine-rich arm (K237–K239).

Early studies done on yeast Hop [1] identified two major and two minor isoforms of the protein with pIs ranging from 5.75 to 6.05, which implied that Hop was differentially phosphorylated. Other studies showed shifts in the isoform composition of Hop to the more acidic range after viral transformation and heat shock [3] and [4]. Immediately upstream of the putative bipartite NLS, two potential phosphorylation sites for casein kinase II (CKII; S189) and cell division cycle 2 (cdc2; T198) kinase were identified using in vitro assays [23]. Despite speculations that Hop is a phosphoprotein, based on indirect evidence, thus far there has been no direct evidence to show that Hop exists as a phosphoprotein in vivo.

There is evidence to suggest that the localization of Hop is regulated by phosphorylation. Hydroxyurea (G1/S arrest) and olomoucine treatment (cdc2 kinase inhibition) of mouse fibroblast cells resulted in cytoplasmic and nuclear localization of Hop [22]. Experiments with phosphorylation mimics of Hop (Hop-S189E and Hop-T198E) suggested that phosphorylation of Hop by cdc2 kinase promoted the cytoplasmic retention of Hop, whereas phosphorylation of Hop by CKII promoted nuclear localization [22]. Both these putative phosphorylation sites were found proximal to the Hsp90-binding TPR2A domain of Hop, suggesting that Hop–Hsp90 interactions may also be modulated by phosphorylation. We also noted that the proposed NLS of Hop overlapped with its TPR2A domain. Moreover residues within the spacer region of the NLS (K229, N233 and K301) were shown to be crucial for Hop–Hsp90 interactions [6] and [15]. Therefore, we hypothesize that the putative bipartite NLS of Hop, and its phosphorylation status, play a significant role in its subcellular localization and association with Hsp90. In this report we have further characterized the putative bipartite NLS of Hop, provided the first direct evidence that Hop was phosphorylated in vivo, showed that heat shock affected the subcellular localization and phosphorylation of Hop, and provided evidence that Hop–Hsp90 interactions involved residues in the putative NLS and may be influenced by phosphorylation.

2. Experimental procedures

2.1. General

Common molecular biology procedures such as agarose gel electrophoresis, ligation reactions, competent bacterial cell preparation, plasmid DNA isolation and restriction enzyme digestion were performed according to standard protocols [24]. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was according to Laemmli [25], and Western blotting according to Towbin et al. [26]. Luminol-based chemiluminescence detection reagents (ECL Advance Western

Blotting Detection Kit; Amersham Biosciences) were used for Western analysis. Protein estimation was done using the 2-D Quant Kit (Amersham Biosciences).

2.2. Site-directed mutagenesis

All mutations were generated from the plasmids pGEX3X2000 and pSK-mSTI1-EGFP, which contain the cDNAs encoding the full-length proteins mSTI1 (mouse homolog of Hop) as a C-terminal fusion with glutathione-*S*-transferase (GST) and mSTI1 as an N-terminal fusion with enhanced green fluorescent protein (EGFP), respectively. All mutations were carried out by site-directed mutagenesis using a double-stranded whole plasmid linear amplification procedure (QuikChange mutagenesis kit; Stratagene). For screening purposes, silent mutations were engineered to create restriction sites except where the desired codon change(s) automatically generated restriction sites. The following mutant proteins were generated: GST-Hop-K239A, GST-Hop/K237/238/239A, GST-Hop-T198E, GST-Hop-S189E, Hop-K239A-EGFP, and Hop-K237/K238/K239A-EGFP. Mutations were confirmed both by restriction enzyme analysis and by DNA sequencing. For ease of manipulation, the mSTI1-EGFP encoding fragment in pSK-mSTI1-EGFP was used as a template for mutagenesis before subcloning into pBCMGSNeo [27] XhoI/NotI sites to produce pB-mSTI1-EGFP and its mutant derivatives, as described previously [22]. Note: All plasmid constructs listed retain their original cloning nomenclature which refers to mSTI1 (mouse homolog of Hop); however the proteins they encode have been labeled Hop for this study so as to limit the number of acronyms used for the same protein.

2.3. Production and purification of GST-Hop fusion proteins

Exponentially growing *Escherichia coli* XLI Blue cells carrying pGEX3X derived plasmid constructs were induced for 5–6 h at 37 °C with 1 mM isopropyl-1-thio-β-d-galactopyranoside (IPTG). The cells were harvested and lysed by mild sonication in 0.01 culture volume of ice-cold phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.3) containing 1 mM final concentration of phenylmethylsulfonyl fluoride (PMSF). The sonicate was incubated, with gentle agitation, for 30 min at room temperature after addition of Triton X-100 to 1% final concentration. The extracts were clarified by centrifugation at 12,000 *g* for 20 min at 4 °C. Aliquots of clarified extracts were added to 50% (w/v) slurry of glutathione-agarose beads (2 mL bed volume per 100 mL of extract) previously equilibrated with PBS. Binding was allowed to occur for 1 h at 4 °C with gentle rocking. The beads were washed extensively with ice-cold PBS before the bound GST fusion proteins were eluted by adding appropriate volume of the elution buffer (10 mM reduced glutathione in 50 mM Tris–HCl, pH 8.0). Eluted GST fusion proteins were

analyzed on SDS-PAGE (12% acrylamide/bis-acrylamide w/v). GST-Hop and its mutant derivatives were all successfully expressed and found to be soluble proteins, which were purified to at least 90% purity.

2.4. Surface plasmon resonance spectroscopy

Surface plasmon resonance (SPR) spectroscopy was performed using a Biacore X apparatus (BIACORE, Sweden). A mixture of NHS/EDC (*N*-hydroxysuccinimide and 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide) was used to activate the surface of a CM5 sensor chip by modification of the carboxymethyl groups to *N*-Hydroxysuccinimide esters. Purified rat liver Hsp90 (untagged) was injected into the first flow cell, such that the *N*-Hydroxysuccinimide esters reacted spontaneously with the amines on the Hsp90 to form covalent links.

The second flow cell was used as a control with no protein immobilized on it. Approximately 9165 response units (RU) of Hsp90 were immobilized onto the chip. After equilibration with running buffer (10 mM Hepes buffer containing 140 mM KCl and 0.001% Tween), increasing concentrations (0.2 μm, 1 μm and 5 μm) of GST-Hop and its derivatives were passed over the immobilized Hsp90 at a flow rate of 10 μL min^{-1}. GST was passed through both cells and any non-specific binding to Hsp90 and/or the chip was monitored. All experiments were performed at room temperature. The CM5 Sensor Chip (Research Grade) was purchased from BIACORE (Sweden). The data was analyzed using the BIAevaluation software version 3.0.

2.5. Hop isoform analysis by two-dimensional gel electrophoresis

To satisfactorily determine the in vivo phosphorylated isoform status of Hop, NIH 3T3 fibroblast cells were treated in various ways prior to preparation of a lysate for analysis by two-dimensional (2D) gel electrophoresis. The cells were either treated with phosphatase inhibitors (PI) okadeic acid (30 nM; specific for serine/threonine phosphatases) and sodium vanadate (2 mM; specific for tyrosine phosphatases), to limit or eliminate any dephosphorylation of Hop, or treated with shrimp alkaline phosphatase (SAP; 10 U) to dephosphorylate any phosphorylated isoforms of Hop. The NIH 3T3 fibroblast cells treated for isoform analysis were harvested by scraping into ice-cold PBS. The cell pellets obtained were resuspended in two-dimensional (2D) lysis buffer (40 mM Tris–HCL, 8 M Urea, 4% Triton X-100, 1 μg/mL aprotinin and 100 μg/mL PMSF) and lysed on ice for 1 h. The lysate was centrifuged at 13,000 rpm for 30 min on a benchtop microfuge to remove insoluble cell debris. Protein concentration was determined using the 2D-Quant kit (Amersham

Biosciences, USA), before the protein samples were precipitated using the 2D clean-up kit (Amersham Biosciences, USA).

For the first dimension separation by isoelectric focusing (IEF), the precipitated total protein samples were diluted in rehydration buffer (9 M Urea, 2% Triton X-100, 2% Ampholytes, 0.002% bromophenol blue, 100 mM DTT) to a final concentration of 1 μg/μL. A total protein sample (120 μg) was then applied onto an immobilized linear pH gradient (IPG; pH 4–7) strip within the chambers of a Zoom® IPGRunner™ Cassette (Invitrogen) and rehydrated overnight as per the manufacturer's instructions. Rehydrated IPG strips were focused by performing IEF in distilled water. The applied electric potential (V) was increased in a step-wise fashion as follows: 200 V (20 min), 450 V (15 min), 750 V (15 min) and 1000 V (120 min), with a total power of 0.1 W $strip^{-1}$ and current, 0.05 mA $strip^{-1}$. The second dimension was resolved by SDS-PAGE (12% acrylamide/bis-acrylamide w/v) at 200 V for 45 min. Western analysis was carried out on the resolved proteins using antibodies specific to Hop (short chain recombinant antibodies made to the C-terminal region of mSTI1, C-334; Antibodies By Design, MorphoSys®; Germany).

2.6. Cell culture, transfections and preparation of cell lysates

Baby Hamster Kidney (BHK-21) cells and NIH 3T3 mouse fibroblast cells were maintained in Dulbecco's modified Eagle's medium (Cambrex, USA) supplemented with 10% calf serum, penicillin (100 U mL^{-1}) and streptomycin (100 U mL^{-1}) (Highveld Biological PTY. Ltd., South Africa) in a humidified atmosphere, at 37 °C with 5% CO_2.

Transient transfections were conducted on BHK-21 cells using IBAfect (for magnet assisted transfection) and MA Lipofectin Enhancer (IBA, Germany) on cells seeded to 60% confluency, according to the manufacturer's instructions. Leptomycin-B (Synexa Life Sciences, South Africa) was used to arrest the nuclear export of proteins; cells were treated with 2 ng mL^{-1} of leptomycin-B overnight at 37 °C.

Cells were fixed in 4% paraformaldehyde for 10 min and nuclei stained with 2 ng mL^{-1} 4′,6-diamidino-2-phenylindole (DAPI; Sigma) in PBS. Slides were mounted in fluorescent mounting solution (DAKO) and the fluorescent images were viewed and captured using the Zeiss LSM 510 Meta confocal microscope (40 × oil immersion objective) and LSM 510 software (Zeiss).

2.7. Biochemical fractionation

BHK-21 cells were chemically fractionated using the ProteoExtract® Subcellular Proteome Extraction Kit (Calbiochem) according to the manufacturer's instructions. Proteins obtained from each fraction were resolved by SDS-PAGE (12% acrylamide/bis-acrylamide w/v), and then subjected to Western analysis, using antibodies specific for β-actin (A-2228, Sigma), Hsp72/73 (SPA-820, StressGen), Hsp40 (Hdj1 homologue; SPA-400, StressGen), Hsp90 ⁎and β (SPA-835, StressGen), and Hop (Antibodies by Design, MorphoSys®; Germany).

3. Results

3.1. Hop was phosphorylated in vivo

Various different lysates were prepared from NIH 3T3 cells treated with phosphatase inhibitors (PI) or a phosphatase (SAP). This allowed the potential phosphorylated state of Hop in vivo to be analyzed after 2D gel electrophoresis and Western analysis for the detection of Hop isoforms (Fig. 1A). Previously published phosphorylation studies of mouse Hop were performed using cell lysates that were treated with a tyrosine phosphatase inhibitor (sodium vanadate) but no serine/threonine phosphatase inhibitors [4]. As preliminary work to the study presented here, the 2D-electrophoretic experiments described by Lässle et al. [4] were repeated but without using any phosphatase inhibitors. The results were identical to those previously obtained [4] suggesting that Hop was phosphorylated at sites other than tyrosine. Thus the present study made use of serine/threonine phosphatase inhibitors, in addition to the tyrosine phosphatase inhibitor.

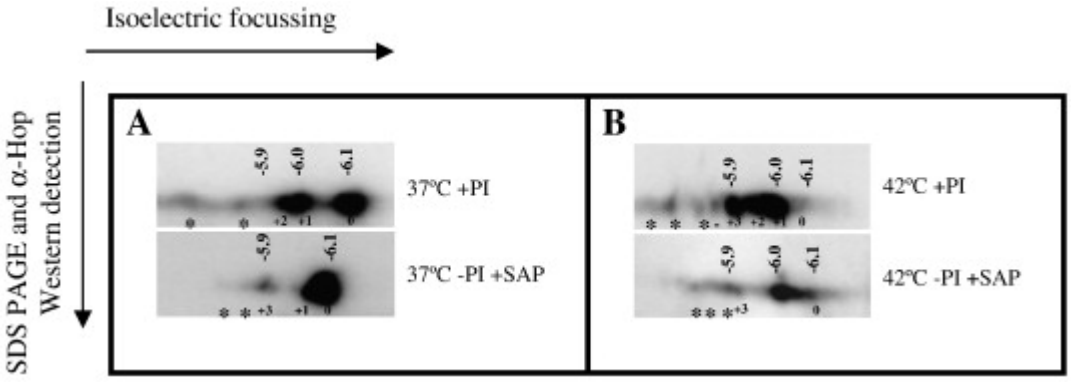


Fig. 1. Hop was differentially phosphorylated in vivo at 37 °C and 42 °C. (A) The isoform composition of Hop after resolution by 2D gel electrophoresis in the pH range 4–7 of 120 μg of total protein extract from NIH 3T3 cells (prepared as described in Experimental procedures), followed by Western analysis for the detection of Hop. The isoelectric focusing (IEF) and SDS-

PAGE steps are shown. The upper panel shows the Western of lysates prepared from cells that were grown under 37 °C and treated with phosphatase inhibitors (37 °C + PI); the lower panel shows the Western of lysates prepared from cells that were also grown at 37 °C and treated with shrimp alkaline phosphatase (SAP) but not treated with PI (37 °C –PI + SAP). Approximate pIs for the prominent isoforms were estimated and are indicated at the top of each panel. The position of the isoforms with assigned pIs did not always coincide due to technical discrepancies such as slight changes in gel size. The major isoform under normal conditions, with an approximate pI of 6.1 was arbitrarily given the label '0'. Any isoform that was found to be more acidic than '0' was labeled with increasing positive numbers. Additional isoforms which were not assigned a pI value were labeled with an asterisk. This figure was representative of the results obtained from at least three independently conducted experiments. (B). The two panels show cell extracts prepared from cells that were incubated for 30 min at 42 °C. The upper panel shows the isoform composition of Hop in cells lysed in the absence of SAP but presence of PI and the lower panel, cells lysed in the presence of SAP. This figure was representative of the results obtained from at least three independently conducted experiments.

For lysates from cells treated with PI and no SAP added, Hop was found to exist not only as a major and a minor isoform, but also as three more relatively acidic isoforms (Fig. 1A, upper panel). The major isoform with a calculated pI of approximately 6.1 was labeled '0' and the minor isoform found at a pI of approximately 6.0 was labeled '+ 1'. A third isoform, approaching a pI of 6.0 and merging with isoform + 1 was labeled '+ 2'. The two other relatively more acidic isoforms at pIs less than 5.9 were designated by asterisks and were not assigned approximate pIs. For lysates from cells treated only with SAP there was a considerable reduction in isoforms + 1 and + 2 (Fig. 1A, lower panel), suggesting that they were dephosphorylated by the SAP treatment. These results provided evidence of in vivo phosphorylation of at least two subpopulations of Hop under normal conditions. The most acidic isoform obtained under normal conditions (designated by an asterisk) disappeared after SAP treatment, indicating that this isoform may have corresponded to a minor phosphorylated isoform of Hop. A prominent isoform, designated '+ 3' was detected after SAP treatment at an approximate pI of 5.9, and this isoform was not prominent in lysate obtained from normally grown cells that were treated with PI (Fig. 1A, upper panel). These results directly related the occurrence of certain acidic isoforms of Hop to post-translational phosphorylation of the protein.

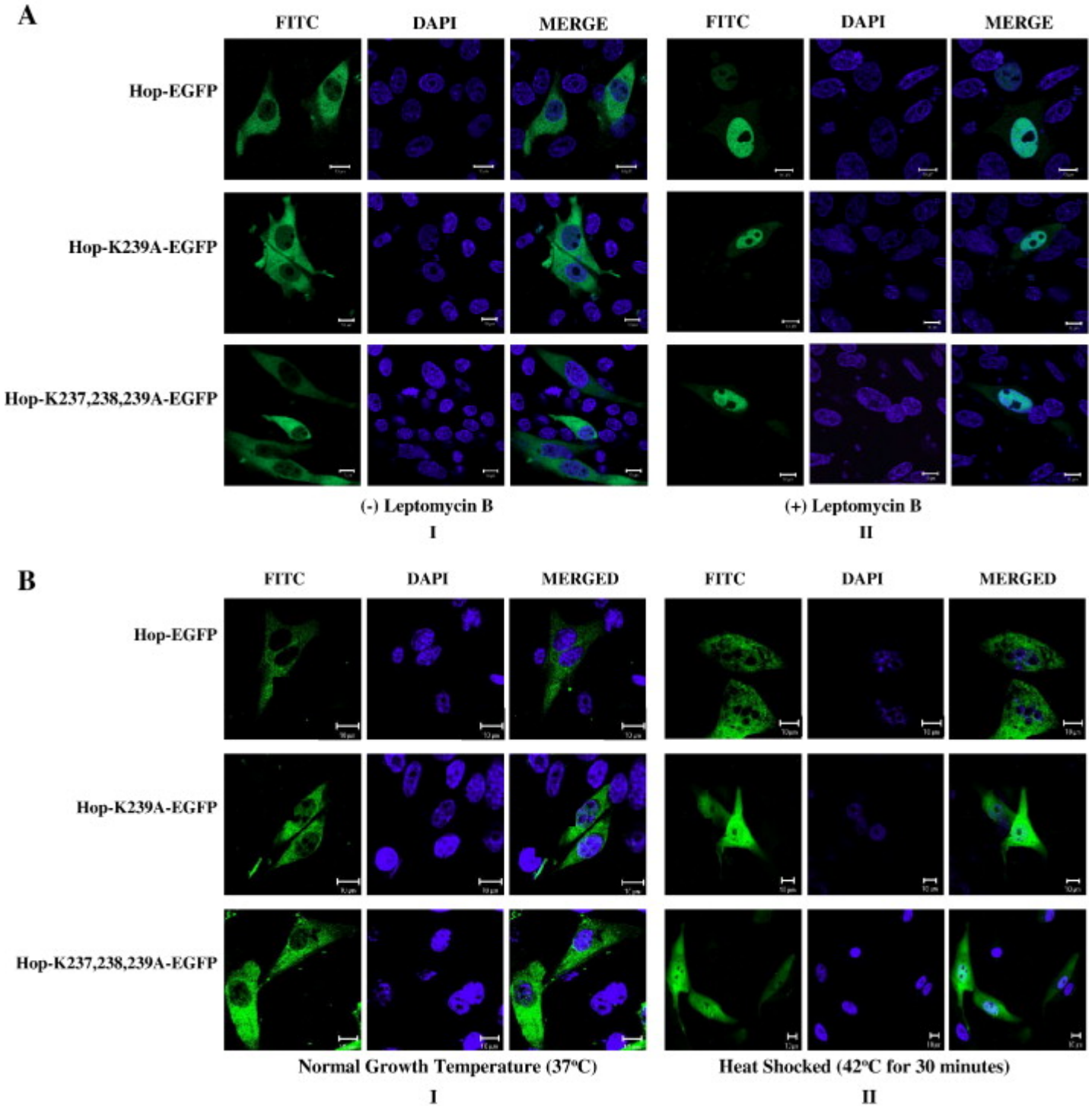
Mouse NIH 3T3 cells were subjected to heat shock, and shown to undergo induction of the stress response as evidenced by induction of Hsp72 protein (data not shown). The isoform composition

of Hop after 30 min of heat shock (Fig. 1B, upper panel) differed from that of normal conditions in that there was a decrease in the amount of the major isoform 0. There was also an increase of a number of minor acidic isoforms, the most prominent of which had an approximate pI of 5.9 and was designated '+ 3'. This isoform was also seen in the SAP treated normal lysate (Fig. 1A, lower panel). The additional minor isoforms that appeared after heat shock were not assigned pIs, and were rather indicated by asterisks. However, it was noted that these isoforms were found at pIs of 5.9 or lower. Lysates prepared after treatment of heat-shocked cells with SAP yielded an isoform profile for Hop (Fig. 1B, lower panel) which included a slight increase of the previously major isoform '0', and the complete absence of isoform + 2. Isoform + 3 showed a considerable reduction after treatment with SAP. These results were consistent with previous studies [4] that showed that heat shock resulted in modifications of Hop. These data also provided evidence for the first time that Hop was phosphorylated in vivo after heat shock.

3.2. The major arm of the proposed bipartite NLS did not contribute to the nuclear localization of Hop under leptomycin-B treatment

We examined the subcellular distribution of Hop in baby hamster kidney cells (BHK-21), using a Hop-EGFP fusion protein, which could also be modified to determine the possible effects of mutations in the predicted NLS. The protein was transiently expressed in BHK-21 cells, fixed, and then analyzed by confocal laser fluorescence microscopy.

Under normal conditions Hop-EGFP was found to localize predominantly to the cytoplasm of most cells (Fig. 2A, I; B, I; C), whereas the localization pattern of EGFP was found to be distributed throughout the cell and therefore identical to previously published findings [data not shown; [4] and [22]]. Treatment of transfected cells with leptomycin-B, a specific inhibitor of the CRM-1 nuclear protein export pathway, showed the accumulation of Hop-EGFP in the nucleus (Fig. 2A II; C), a finding that correlates with previous literature [22].


A
FITC
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(-) Leptomycin B
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II
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FITC
DAPI
MERGED
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Hop-K237,238,239A-EGFP
Normal Growth Temperature (37ºC)
I
Heat Shocked (42ºC for 30 minutes)
II

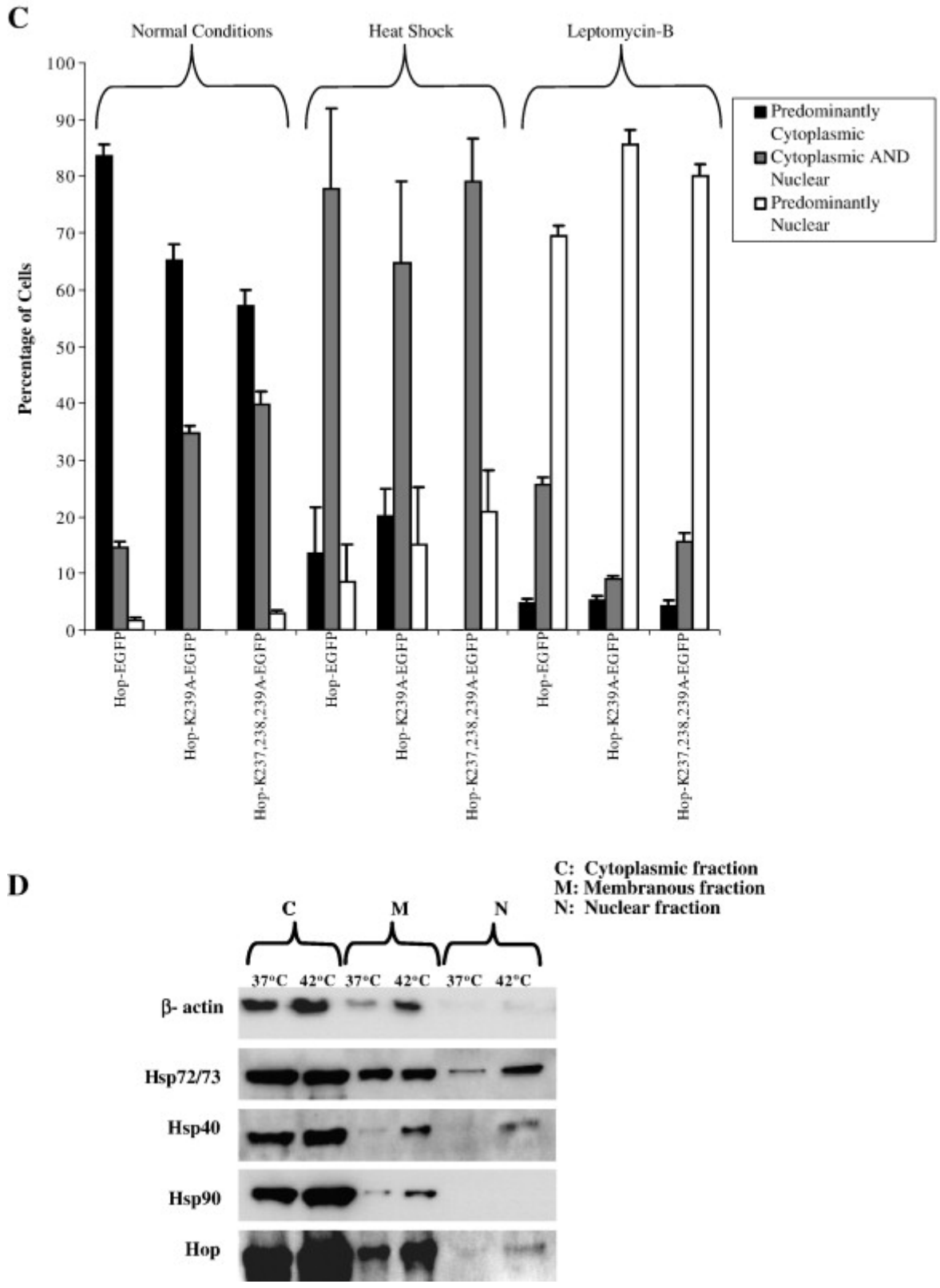

Fig. 2. Hop was localized to the nucleus under leptomycin-B treatment and heat shock conditions; the nuclear localization of Hop was not dependent on the major arm of the NLS. (A) BHK-21 cells were transiently transfected with pB-mSTI1-EGFP (encoding Hop-EGFP) and two Hop-NLS mutants, pB-mSTI1-K239A-EGFP (encoding Hop-K239A-EGFP) and pB-mSTI1-K237/238/239A-

EGFP (encoding Hop-K237/238/239A-EGFP), and grown at 37 °C. Cells were treated with leptomycin-B, fixed, stained with DAPI (blue), mounted and then visualized by confocal laser fluorescence microscopy. The labels on top of each panel refers to the filters used for visualization on the confocal microscope (FITC for EGFP, DAPI for nuclei, and MERGED). Scale bars, 10 μm. (B). BHK-21 cells grown and transiently transfected in the same manner described above, were subjected to acute heat shock by incubation with pre-warmed media 30 min at 42 °C. The cells were then fixed and visualized as described above. Scale bars, 10 μm. (C) BHK-21 cells were transiently transfected and treated as mentioned in A and B. Cells showing predominantly cytoplasmic fluorescence, cytoplasmic and nuclear fluorescence and predominantly nuclear fluorescence were quantified. Average values were taken from six different fields and the error bars represent standard deviations. (D) Western analysis using antibodies against β-actin (cytoplasmic and membrane marker), Hsp72/73, Hsp40, Hsp90 and Hop, after the biochemical fractionation of whole BHK-21 cell lysate obtained from cells grown at 37 °C and cells incubated for 30 min at 42 °C. Identical cell numbers were processed at 37 °C and 42 °C, and volume equivalence of each subcellular fraction was loaded proportional to the identical number of cells they were extracted from. The results shown are representative of three independent experiments.

We mutated the residues of the major arm of the proposed NLS, a region in most bipartite NLSs that is critical for functionality in vivo [28], [29] and [30], and examined the subcellular localization patterns of the derivative proteins. Both NLS mutants of Hop-EGFP also showed a predominantly cytoplasmic profile of subcellular localization under normal growth conditions (Fig. 2A, I; B, I). However, closer inspection of the various localization patterns by quantitative analysis revealed that both of the mutant Hop-EGFP derivatives showed a greater incidence of cytoplasmic and nuclear localization compared to unmutated Hop-EGFP (Fig. 2C). Leptomycin-B treated cells showed that both mutant Hop-EGFP derivatives were ‘arrested’ within the nucleus, as was the case for unmutated Hop-EGFP (Fig. 2A, II; C).

3.3. Hop was localized to the nucleus under heat shock and the nuclear localization of Hop was not dependent on the major arm of the proposed NLS

BHK-21 cells were treated with pre-warmed DMEM and heat shocked at 42 °C for 30 min after transient transfection. In response to heat shock, Hop and its putative NLS mutants showed increased nuclear localization compared to its subcellular localization under normal conditions (Fig. 2B I and II; C).

Total cell extracts obtained from cells grown at 37 °C and cells heat shocked at 42 °C for 30 min, were biochemically fractionated into cytoplasmic, membrane and nuclear fractions. Volume equivalence of each subcellular fraction was loaded proportional to the identical number of cells they were extracted from. The proteins in each fraction were resolved by SDS-PAGE and then subjected to Western blot analysis using antibodies against β-actin, Hsp72/73, Hsp40, Hsp90 and Hop (Fig. 2D). β-actin was found, as expected, in high amounts in the cytoplasmic and membranous extracts of both normal and heat-shocked extracts (Fig. 2D, Panel 1: lanes 1, 2, 3 and 4). No β-actin was detected in the nuclear fraction from cells grown under normal conditions (Fig. 2D, Panel 1: lane 5), suggesting that this fraction was relatively free of contamination from the cytoplasmic fraction. Although generally not nuclear, β-actin is known to reversibly localize to the nucleus under heat shock [31] and [32] and a faint band was thus visible in the nuclear extract of heat-shocked extracts (Fig. 2D, Panel 1: lane 6). Hsp72/73 was found to be predominantly cytoplasmic and membranous under normal and heat shock conditions (Fig. 2D, Panel 2: lanes 1–4). There was a slight presence of Hsp72/73 in the nuclear fractions before heat shock and a definite nuclear fractionation of Hsp72/73 under heat shock (Fig. 2D, Panel 2: lanes 5–6). The detection of Hsp72/73 in the nuclear fraction after heat shock was consistent with what has been shown previously [33], and these data together with the actin data, suggested that the nuclear fractions had been correctly isolated.

The localization of Hsp40 (Hdj1 homologue) was also analyzed, since it is a co-chaperone of Hsp70, and like Hsp70 translocates from the cytoplasm to the nucleus under heat shock conditions [34]. Hsp40 was detected in the cytoplasmic fractions under normal and heat-shocked conditions, as was expected, with an increased detection in the membranous fraction after heat shock (Fig. 2D, Panel 3: lanes 1–4). Although Hsp40 could not be detected in the nuclear fractions obtained from normally grown cells, it was visibly co-fractionated with Hsp70 within the nuclear proteins from heat-shocked cells (Fig. 2D, Panel 3: lanes 5–6). Hsp90 was detected predominantly in the cytoplasmic fraction, with an increased detection in the membranous fraction after heat shock (Fig. 2D, Panel 4: lanes 1–4). Although previous reports have shown the nuclear translocation of Hsp90 during stress conditions [5], [35] and [36], Hsp90 was not detected in the heat-shocked nuclear fractions obtained in this study (Fig. 2D, Panel 4: lanes 5–6). Hop also showed predominantly cytoplasmic fractionation under normal and heat shock conditions with some detection in the membranous fraction. This increased after heat shock (Fig. 2D, Panel 5: lanes 1–4). The detection of Hop was evident in the nuclear fraction of heat-shocked lysate (Fig. 2D, Panel 5: lane 6). Taken together these results demonstrated that a population of Hop

localized to the nucleus when the cells were subjected to heat shock, and that mutations of the major arm of the proposed NLS of Hop did not disrupt this nuclear localization.

3.4. Proposed NLS residues were critical for Hop–Hsp90 interactions and a cdc2 kinase phosphorylation mimic of Hop reduced its affinity for Hsp90

SPR was used to investigate Hop–Hsp90 interactions when one or more residues of the major arm of the proposed NLS of Hop were mutated. Hsp90 was immobilized on the sensor chip and GST-Hop constructs were passed over the chip. GST was used as a negative control to monitor any non-specific binding of GST-Hop and its derivatives to Hsp90. Affinity constants in the form of K_D values were determined for the binding of GST-Hop to Hsp90 and found to be 1.4 μM, consistent with previously published data [6] and [15]. Both GST-Hop-K239A and GST-Hop-K237/238/239A showed abrogated binding to Hsp90 and were comparable to the negative control GST (Fig. 3A). These results suggested that residues K237, K238 and K239 were critical for the interaction of Hop with Hsp90. A three dimensional (3D) representation of the structure of the TPR2A domain of Hop interacting with the C-terminal MEEVD pentapeptide of Hsp90 (Fig. 3B) showed that K237, K238 and K239 potentially made contact with the methionine of the Hsp90 MEEVD motif.

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We mutated the residues of the major arm of the proposed NLS, a region in most bipartite NLSs that is critical for functionality in vivo [28], [29] and [30], and examined the subcellular localization patterns of the derivative proteins. Both NLS mutants of Hop-EGFP also showed a predominantly cytoplasmic profile of subcellular localization under normal growth conditions (Fig. 2A, I; B, I). However, closer inspection of the various localization patterns by quantitative analysis revealed that both of the mutant Hop-EGFP derivatives showed a greater incidence of cytoplasmic and nuclear localization compared to unmutated Hop-EGFP (Fig. 2C). Leptomycin-B treated cells showed that both mutant Hop-EGFP derivatives were 'arrested' within the nucleus, as was the case for unmutated Hop-EGFP (Fig. 2A, II; C).

3.3. Hop was localized to the nucleus under heat shock and the nuclear localization of Hop was not dependent on the major arm of the proposed NLS

BHK-21 cells were treated with pre-warmed DMEM and heat shocked at 42 °C for 30 min after transient transfection. In response to heat shock, Hop and its putative NLS mutants showed increased nuclear localization compared to its subcellular localization under normal conditions (Fig. 2B I and II; C).

Total cell extracts obtained from cells grown at 37 °C and cells heat shocked at 42 °C for 30 min, were biochemically fractionated into cytoplasmic, membrane and nuclear fractions. Volume equivalence of each subcellular fraction was loaded proportional to the identical number of cells they were extracted from. The proteins in each fraction were resolved by SDS-PAGE and then subjected to Western blot analysis using antibodies against β-actin, Hsp72/73, Hsp40, Hsp90 and Hop (Fig. 2D). β-actin was found, as expected, in high amounts in the cytoplasmic and membranous extracts of both normal and heat-shocked extracts (Fig. 2D, Panel 1: lanes 1, 2, 3 and 4). No β-actin was detected in the nuclear fraction from cells grown under normal conditions (Fig. 2D, Panel 1: lane 5), suggesting that this fraction was relatively free of contamination from the cytoplasmic fraction. Although generally not nuclear, β-actin is known to reversibly localize to the nucleus under heat shock [31] and [32] and a faint band was thus visible in the nuclear extract of heat-shocked extracts (Fig. 2D, Panel 1: lane 6). Hsp72/73 was found to be predominantly cytoplasmic and membranous under normal and heat shock conditions (Fig. 2D, Panel 2: lanes 1–

4). There was a slight presence of Hsp72/73 in the nuclear fractions before heat shock and a definite nuclear fractionation of Hsp72/73 under heat shock (Fig. 2D, Panel 2: lanes 5–6). The detection of Hsp72/73 in the nuclear fraction after heat shock was consistent with what has been shown previously [33], and these data together with the actin data, suggested that the nuclear fractions had been correctly isolated.

The localization of Hsp40 (Hdj1 homologue) was also analyzed, since it is a co-chaperone of Hsp70, and like Hsp70 translocates from the cytoplasm to the nucleus under heat shock conditions [34]. Hsp40 was detected in the cytoplasmic fractions under normal and heat-shocked conditions, as was expected, with an increased detection in the membranous fraction after heat shock (Fig. 2D, Panel 3: lanes 1–4). Although Hsp40 could not be detected in the nuclear fractions obtained from normally grown cells, it was visibly co-fractionated with Hsp70 within the nuclear proteins from heat-shocked cells (Fig. 2D, Panel 3: lanes 5–6). Hsp90 was detected predominantly in the cytoplasmic fraction, with an increased detection in the membranous fraction after heat shock (Fig. 2D, Panel 4: lanes 1–4). Although previous reports have shown the nuclear translocation of Hsp90 during stress conditions [5], [35] and [36], Hsp90 was not detected in the heat-shocked nuclear fractions obtained in this study (Fig. 2D, Panel 4: lanes 5–6). Hop also showed predominantly cytoplasmic fractionation under normal and heat shock conditions with some detection in the membranous fraction. This increased after heat shock (Fig. 2D, Panel 5: lanes 1–4). The detection of Hop was evident in the nuclear fraction of heat-shocked lysate (Fig. 2D, Panel 5: lane 6). Taken together these results demonstrated that a population of Hop localized to the nucleus when the cells were subjected to heat shock, and that mutations of the major arm of the proposed NLS of Hop did not disrupt this nuclear localization.

3.4. Proposed NLS residues were critical for Hop–Hsp90 interactions and a cdc2 kinase phosphorylation mimic of Hop reduced its affinity for Hsp90

SPR was used to investigate Hop–Hsp90 interactions when one or more residues of the major arm of the proposed NLS of Hop were mutated. Hsp90 was immobilized on the sensor chip and GST-Hop constructs were passed over the chip. GST was used as a negative control to monitor any non-specific binding of GST-Hop and its derivatives to Hsp90. Affinity constants in the form of K_D values were determined for the binding of GST-Hop to Hsp90 and found to be 1.4 μM, consistent with previously published data [6] and [15]. Both GST-Hop-K239A and GST-Hop-K237/238/239A showed abrogated binding to Hsp90 and were comparable to the negative control GST (Fig. 3A). These results suggested that residues K237, K238 and K239 were critical for the interaction of Hop with Hsp90. A three dimensional (3D) representation of the structure of the

TPR2A domain of Hop interacting with the C-terminal MEEVD pentapeptide of Hsp90 (Fig. 3B) showed that K237, K238 and K239 potentially made contact with the methionine of the Hsp90 MEEVD motif.

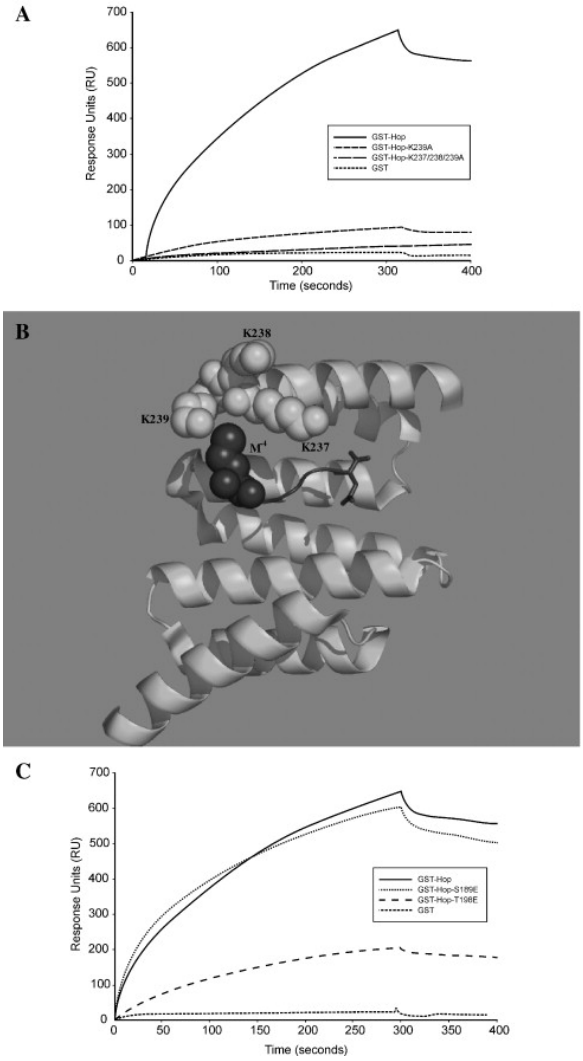

Fig. 3. Proposed NLS residues were critical for Hop–Hsp90 interaction and the phosphorylation of Hop by cdc2 kinase may perturb Hop–Hsp90 interactions. (A) Binding response curves of GST-Hop and its NLS mutants (0.2–5 μM used, only 5 μM trace shown for each; GST-Hop-K239A and GST-Hop-K237/238/239A) to Hsp90 (immobilized) were generated using surface plasmon resonance spectroscopy (SPR). (B) Ribbon representation of the structure of TPR2A of Hop (light grey) with the C-terminal MEEVD motif of Hsp90 shown in dark grey (1ELR; [6]). The space filled residues, K237, K238 and K239 form the major arm of the predicted NLS, and these are shown relative to the space filled residue, methionine, which forms part of the pentapeptide MEEVD of Hsp90. This figure was generated using Pymol Molecular Graphics Software [[49]; http://pymol.sourceforge.net]. (C) Binding response curves of GST-Hop and its phosphorylation

mimics (GST-Hop-S189E, mimic of phosphorylation by CKII kinase; and GST-Hop-T198, mimic of phosphorylation by cdc2 kinase) to Hsp90 using SPR. GST was used as a control for non-specific binding in A and C.

We also investigated the effect of phosphorylation at sites S189 and T198 of Hop on its interactions with Hsp90. Phosphorylation was mimicked at both these sites by substituting the respective serine and threonine residues with an acidic residue, glutamic acid. SPR analysis was conducted to determine the extent of interaction of both these Hop phosphorylation mimics with Hsp90. The binding curves indicated that the CKII kinase phosphorylation mimic of Hop (GST-Hop-S189E) did not show much deviation from the unmutated GST-Hop (Fig. 3C), whereas the cdc2 kinase phosphorylation mimic of Hop (GST-Hop-T198E) exhibit diminished affinity for Hsp90. K_D values of 2.5 μM and 1 μM were obtained for GST-Hop-T198E–Hsp90 and GST-Hop-S189E–Hsp90 interactions respectively. These values may or may not be of biological significance in vivo.

All SPR analyses were repeated independently by immobilizing the GST-Hop constructs on a sensor chip and passing over purified Hsp90. Similar results were obtained (data not shown). Taken together, the results from these experiments showed that the major arm of the proposed NLS was crucial for Hop–Hsp90 interactions, and that mimicking phosphorylation by cdc2 kinase reduced the affinity of Hop for Hsp90.

4. Discussion

This is the first report to have provided direct evidence of Hop being a phosphoprotein under normal and stress conditions, and that the subcellular localization of Hop was primarily stress regulated. We have established that unlike previously reported bipartite NLSs, the major arm of the proposed NLS of Hop was not critical for its nuclear translocation. However these residues were crucial for Hop–Hsp90 interaction, and through this interaction, we hypothesize that this region could possibly serve to retain Hop within the cytoplasm. Furthermore, phosphorylation-mimic studies suggested that phosphorylation of Hop at a cdc2 kinase site may diminish the affinity of Hop for Hsp90, whilst phosphorylation of Hop at a CKII site may not interfere with Hop–Hsp90 interactions [22].

The presence of okadeic acid (serine/threonine phosphatase inhibitor) in the phosphorylation study conducted, suggested that phosphorylation of Hop occurred predominantly at serine or threonine residues. This is further substantiated by evidence of in vitro phosphorylation of Hop at S189 and T198 [22] and [23]. Heat stress may exert inhibitory and activating effects on various

kinases, including Mitogen-activated protein (MAP) kinases, and phosphatases [37], [38], [39] and [40]. Hop was found to have a potential phosphorylation site for MAPKAP kinase 2 and two potential sites for S6 kinases within its amino acid sequence [4]. Hop may thus be phosphorylated by a stress kinase pathway in vivo [4] in response to heat shock. The differentially phosphorylated isoforms of Hop may correspond to subpopulations of Hop, which are localized to varying subcellular compartments during normal and/or stress conditions. Since Hop is known to be predominantly cytoplasmic under normal conditions in this study and in others [4] and [22], the major '0' isoform of Hop resolved by 2D analysis, may correspond to the cytoplasmic population of Hop under normal conditions.

Acute heat shock resulted in increased incidence of cells showing greater translocation of Hop-EGFP to the nucleus (but not nucleolus). This was supported by the detection of endogenous Hop in the nuclear protein fractions after heat shock. Short-term heat shock in mammalian cells is known to cause the transient arrest of the cell cycle at the G1/S stage [41]. The arrest of mouse fibroblast cells in the G1/S phase showed increased nuclear localization of Hop [22]. Since the heat shock conditions used here also increased the localization of Hop to the nucleus, these conditions probably promoted G1/S arrest of the cells. The nuclear localization of Hop may thus be primarily stress regulated and secondarily regulated by cell cycle events.

Although mutations within the major arm of the putative NLS of Hop did not disrupt the nuclear translocation of the Hop-EGFP construct, SPR analysis showed that these mutations resulted in minimal interaction of GST-Hop with Hsp90. A three-dimensional (3D) representation of the Hop TPR2A domain showing the NLS residues that were mutated, together with the C-terminal pentapeptide of Hsp90 (MEEVD motif), illustrated the proximity of these NLS residues to the methionine of the Hsp90 peptide (Fig. 3B). One or more of these residues could be involved in direct interactions with Hsp90.

We propose that the minor arm of this putative NLS could be the functional NLS, acting together with a second putative monopartite NLS found within amino acids 337–351 (Fig. 4). Mutation of the major arm of the putative bipartite NLS may have resulted in a modification of the structure of Hop such that the putative monopartite NLS was better positioned and exposed to importin binding, thereby facilitating more efficient nuclear localization than that obtained for unmutated Hop. A second possibility is that the major arm of the putative bipartite NLS of Hop might promote the cytoplasmic retention of Hop by binding to Hsp90, thus explaining why mutation of residues within Hop that rendered it incapable of interacting with Hsp90 in vitro, allowed for greater nuclear

translocation of Hop in vivo. Hsp90 has previously been reported to sequester the glucocorticoid receptor to the cytoplasm through their interaction within a complex [42].

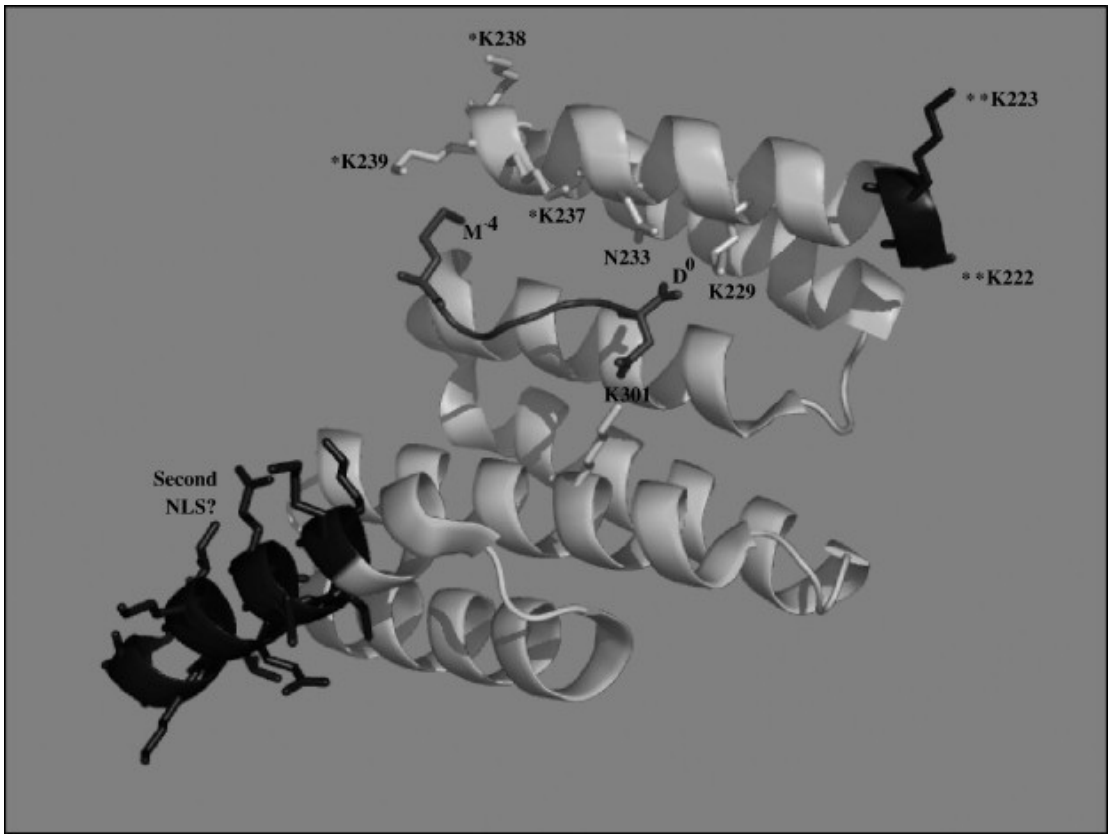

Fig. 4. Positioning of the proposed bipartite NLS with respect to TPR2A domain and a second potential NLS. Ribbon representation of the structure of TPR2A of Hop and a flanking C-terminal helix interacting with the C-terminal MEEVD motif of Hsp90 (1ELR; [6]). The residues, K229, N233 and K301 are those that are known to be critical for interaction with Hsp90. Residues denoted with a single asterisk correspond to those that form the major arm of the bipartite NLS (K237, K238 and K239). The residues denoted with a double asterisk, K222 and K223 (dark grey), form part of the minor arm of this NLS, which may be responsible for functionality of the NLS within the protein. A second NLS has been identified within the flanking C-terminal portion of Hop (337–351) shown in dark grey, which may be working in concert with the first predicted NLS. This figure was generated using Pymol Molecular Graphics Software [49]; http://pymol.sourceforge.net.

A subpopulation of endogenous Hop was detected in the membranous fractions under normal and heat shock conditions. This finding was consistent with reports of the interaction of Hop with Prp^c at the plasma membrane [20]. Interestingly, it was also found that Prp^c interacts with Hop at residues 230–245 [43], which overlaps with the Hsp90 binding site [[15]; and this study]. Prp^c binding to Hop at this site may act as a mechanism to improve the efficiency of retaining a

subpopulation of Hop at the plasma membrane. This further supports the proposed role of Hop–Hsp90 interactions in modulating the subcellular localization of Hop. Our data also showed the co-fractionation of Hsp70, Hsp90 and Hsp40, along with Hop, in the membranes under normal and heat shock conditions. This also suggests that Hop may associate with one or more of its chaperone partners within various membranous structures.

Based on the results obtained from this study, together with what is currently known about Hop particularly regarding its nucleo-cytoplasmic shuttling properties, we propose a model detailed in Fig. 5. Within the normal cytoplasmic environment, Hop equilibrates between an Hsp90-complexed state and an uncomplexed free state [44] and [45], and the equilibrium between the two states may be regulated through phosphorylation of Hop (Fig. 5, arrow A). Phosphorylation of Hop by cdc2 kinase may disrupt Hop–Hsp90 interactions as indicated by the SPR analysis. This frees Hop, but retains it within the cytoplasm [[22]; Fig. 5, arrow C/cdc2?] so that it can interact with other proteins in complexes that are independent of Hsp90 [46] and [47].

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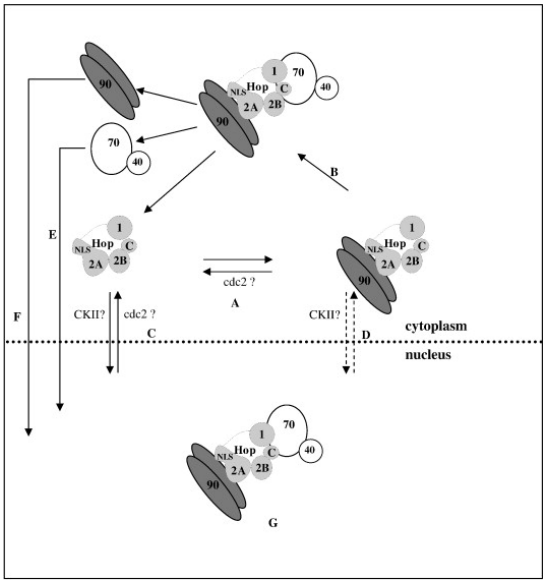

Fig. 5. A model reflecting the possible links between the subcellular localization of the phosphoprotein Hop and its functionality as a Hsp70/Hsp90 scaffolding protein. Hop exists on its own or in complex with Hsp90, in the cytoplasm under normal conditions. This may be regulated by phosphorylation, with cdc2 kinase phosphorylation of Hop disrupting its interaction with Hsp90 (cdc2? arrow; A). Interaction of Hop with Hsp90 is known to facilitate a number of other interactions, of which the most well established one is the interaction of the Hop–Hsp90 complex with Hsp70 (associated with its co-chaperone Hsp40 and substrate) in order to facilitate substrate transfer from Hsp70 to Hsp90 (B). This multichaperone complex then dissociates, freeing its various components. Hop is known to translocate to the nucleus (C) under stressful conditions, and its localization may be regulated by phosphorylation, with CKII phosphorylation possibly promoting nuclear localization (CKII? arrow) and cdc2 kinase phosphorylation possibly promoting cytoplasmic retention (cdc2? arrow). It is speculated that Hop may also be capable of moving into the nucleus in concert with Hsp90 (D) as a complex (arrows shown in dotted lines) by either the putative NLS (222–239), or through the functioning of multiple NLSs, and possibly also promoted

by CKII phosphorylation (CKII? dotted arrow). It is already known that both Hsp70 (together with Hsp40) and Hsp90 translocate into the nucleus under heat shock (E and F respectively). Within the nucleus Hop may have a number of functions, including its basic function of interacting with Hsp70 and/or Hsp90 to form nuclear complexes (G). The 1, 2A, 2B, C and NLS annotations on Hop refer to its TPR1, TPR2A and TPR2B domains, C-terminal domain, and nuclear localization signal sequence, respectively. Hsp40, Hsp70 and Hsp90 are labeled as 40, 70 and 90, respectively.

Under normal conditions, Hop–Hsp90 interactions within the cytoplasm may serve to anchor Hop to this subcellular compartment. The major arm of the proposed NLS of Hop may contribute to the overall localization of Hop by its interaction with Hsp90. This facilitates the assembly of the Hsp70–Hsp90 chaperone complex, which has been shown to be cytoplasmic (Fig. 5, arrow B). It also implies that Hop translocates into the nucleus when it is not complexed with Hsp90, possibly promoted by CKII phosphorylation of Hop (Fig. 5, arrow C/CKII?). Previous reports have shown the nuclear translocation of Hsp90 during stress conditions including heat shock [5], [35] and [36]. In the present study Hsp90 was not detected in the nuclear fractions under normal or heat shock conditions. Biochemical fractionation techniques are known to be disruptive and may have altered the subcellular localization pattern of a protein such as Hsp90, whose translocation into the nucleus may be transient or dynamic in nature. This may also explain why relatively low levels of Hop were detected in the nuclear fractions, when compared to the confocal microscopic data. Thus, if Hsp90 does indeed translocate into the nucleus during heat shock, the absence of functional NLSs within Hsp90 [36] and [48] suggests that Hsp90 may have to “piggyback” on an NLS-bearing protein. CKII phosphorylation was shown to promote nuclear localization of Hop [22] and now, evidence has been presented to suggest that CKII phosphorylation does not disrupt Hop–Hsp90 binding. Thus we cannot exclude the possibility of a subpopulation of Hop–Hsp90 translocating as a complex into the nucleus under specific regulatory conditions, such as phosphorylation by CKII (Fig. 5, dotted arrow D/CKII?). Other studies [34] and ours have shown the presence of Hsp70 and Hsp40 in heat-shocked nuclear fractions. This is suggestive of the nuclear assembly of the Hsp70/Hsp90 chaperone heterocomplex during heat stress (Fig. 5 arrows E, F and G).

The details of the co-localization of Hop and Hsp90, as well as the scaffolding properties of Hop within the nucleus, under conditions of heat shock remains to be investigated. Changes in the ionic state and conformation of Hop due to phosphorylation at S189 and T198, may have an effect on its interactions with other members of the chaperone heterocomplex, such as Hsp70, and this

also needs to be investigated. The mechanism of nuclear localization of the Hsp70–Hop–Hsp90 chaperone heterocomplex under both normal and stress conditions is complex. However, the putative phosphorylation-regulated NLS of Hop appears to play an important role in the functionality of the chaperone heterocomplex, not only on the level of its subcellular localization but also with regards to the assembly of the chaperone heterocomplex.

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