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Stress-induced interaction between p38 MAPK and HSP70

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ABSTRACT

p38 MAPK, one of the four MAPK subfamilies in mammalian cells, is activated by environmental stresses and pro-inflammatory cytokines, playing fundamental roles in many biological processes. Despite all that is known on the structure and functions of p38, many questions still exist. The coupling of activation and nuclear translocation represents an important aspect of p38 signaling. In our effort in exploring the potential chaperone for p38 translocation, we performed an endogenous pull-down assay and identified HSP70 as a potential interacting protein of p38. We confirmed the interaction between p38 and HSP70 *in vitro* and *in vivo*, and identified their interaction domains. We also showed stress-induced nuclear co-localization of these two proteins. Our preliminary result indicated that HSP70 was related to the phosphorylation of MK2, a specific nuclear downstream target of p38, suggesting HSP70 is a potential chaperone for the nuclear translocation of p38.

1. Introduction

The p38 mitogen-activated protein kinase (MAPK) pathway plays a fundamental role in a cell's response to diverse extracellular stimuli, such as growth factors, pro-inflammatory cytokines, and environmental stresses, being involved in the regulation of cell growth, cell differentiation, cell cycle and death, and inflammation [1–6]. So far, four p38 subfamily members, p38(α), p38β, p38γ, and p38δ, have been identified in mammalian cells [1,7,8]. p38 mediates stress signaling via dual phosphorylation of the Thr180 and Tyr182 in the conserved TGY motif by its upstream MAP kinase kinase 3/6 (MKK3/6) [1,2]. Once activated, p38 exerts its multiple functions through various downstream targets including protein kinases (MAPK-activated protein kinase 2/3, MK2/3; MAPK-interacting kinase 1/2, MNK1/2; p38-regulated/activated protein kinase, PRAK/MK5; etc.) and transcription factors (activating transcription factor-2, ATF-2; CHOP/GADD153; myocyte enhancer factor 2, MEF2; Elk-1; p53; etc.) [1,2,9,10].

In spite of numerous studies have been conducted to dissect p38 signaling pathway, and its structure and functions are relatively widely known, some basic questions on p38 that needs to be answered still exist [2]. For instance, p38 resides in the cytoplasm of resting cells, and translocates into the nucleus upon stress-induced activation, yet the underlying mechanisms remain largely unknown [11]. The coupling of activation and nuclear translocation represents an important aspect of p38 MAPK. In our previous study, we showed that stress-induced nuclear accumulation of p38 is a phosphorylation-dependent, microtubule- and dynein-associated process [11]. The most probable manner by which p38 translocates into the nucleus in mammalian cells seems to be via a NLS-containing chaperone, which needs further investigation.

To explore the potential chaperone, we performed an endogenous pull-down assay. Heat-shock cognate 70 (HSC70), a constitutively-expressed heat-shock protein of HSP70 family, was identified as a stress-induced interacting protein of p38 by mass spectrometry. We confirmed the direct association between p38 and HSC70 *in vitro*, and the *in vivo* interaction of p38 with HSC70, and its inducible isoform HSP72 as well. In this report, the terms HSC70 and HSP72 refer to the constitutive and the inducible isoforms, respectively, while HSP70 refers both of them. Then we identified the binding domains of HSC70 to p38. We also showed stress-induced nuclear co-localization of HSP70 and p38, both of which translocated from the cytosol. Our preliminary results indicated the potential role of HSP70 in p38's nuclear function. Taken together, we found that as an interacting protein of p38, HSP70 is a potential chaperone for stress-induced translocation of p38.

2. Materials and methods

2.1. Constructs and siRNAs

cDNAs encoding human HSC70 (GenBank accession number NM_153201) and HSP72 (GenBank accession number NM_005345) were obtained from a human liver cDNA library by PCR, and were

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cloned into a revised pcDNA3 vector which carried an HA tag [10]. FLAG-tagged p38(WT), p38(AF), p38(KM), and His-tagged p38(WT) were constructed as described previously [7,8,11]. GST-HSC70 was obtained by subcloning of HSC70 cDNA into pGEX-4T-1 vector. HA-tagged different truncate forms of HSC70, including HSC70(AD), HSC70(PBD + VD), HSC70(PBD), and HSC70(VD), were constructed by PCR. The primers used were synthesized by Invitrogen (Shanghai, China) and listed in Supplementary Table 1. All the constructs were finally confirmed by sequencing (Invitrogen, Shanghai, China). siRNAs targeting HSC70 were synthesized by GenePharma (Shanghai, China) and the targeting sequences were: HSC70 siRNA1 (5′-CCGAACCACUCCAAGCUAU-3′); HSC70 siRNA2 (5′-CUGUCCUCAUCAAGCGUAA-3′).

2.2. Reagents and antibodies

Ni^{2+}-NTA resin and Glutathione Sepharose 4B beads were products of Qiagen (Germany) and GE Healthcare (USA), respectively. Anti-FLAG-coupled agarose (M2) beads, platelet-derived growth factor (PDGF), and 4,6-diamidino-2-phenylindole (DAPI) were purchased from Sigma–Aldrich (St Louis, USA). Antibodies were from Cell Signaling Technology (p38 MAPK (#9212), phospho-p38 MAPK(#9211), Sepharose beads-conjugated phospho-p38 MAPK (#9219), MK2 (#3042), phospho-MK2 (#3007), HA-tag (#2367), β-actin (#4967), horseradish peroxidase (HRP)-labeled secondary antibodies), Santa Cruz (HSP70 (#sc-24), HSC70 (#sc-1059), His-tag (#sc-803)), Stressgen (HSP72(#SPA-810), and Stratagene (FLAG-tag (#200471)). Alexa Fluor 488-conjugated anti-rabbit and Alexa Fluor 594-conjugated anti-mouse secondary antibodies were obtained from Molecular Probes/Invitrogen (USA).

2.3. In Vitro binding assay

GST and GST-HSC70 were purified with Glutathione Sepharose 4B beads, and His-p38 protein was purified with Ni^{2+}-NTA, following the manufacturers' protocols. Equal amounts of GST and GST-HSC70 were incubated with His-p38 in the GST protein binding buffer (50 mM Tris.Cl, pH8.0, 1 mM EDTA, 100 mM NaCl, 1% Triton X-100, 5 mM DTT) for 3 h on a rotary shaker at 4 °C. After washing three times with binding buffer, the beads were boiled with 1× SDS loading buffer (50 mM Tris–HCl, pH 6.8, 2% SDS, 0.1% bromophenol blue, 10% glycerol, 100 mM DTT), and subjected to 10% SDS–PAGE.

2.4. Cell culture and transfection

COS-7 and HeLa cells were maintained in Dulbecco's Modified Eagle Medium (DMEM, GIBCO BRL/Invitrogen, USA) supplemented with 5% fetal bovine serum (FBS, Hyclone, USA) in the cell incubator (37 °C, 5% CO_2). For plasmid transfection, 5×10^5 COS-7 cells

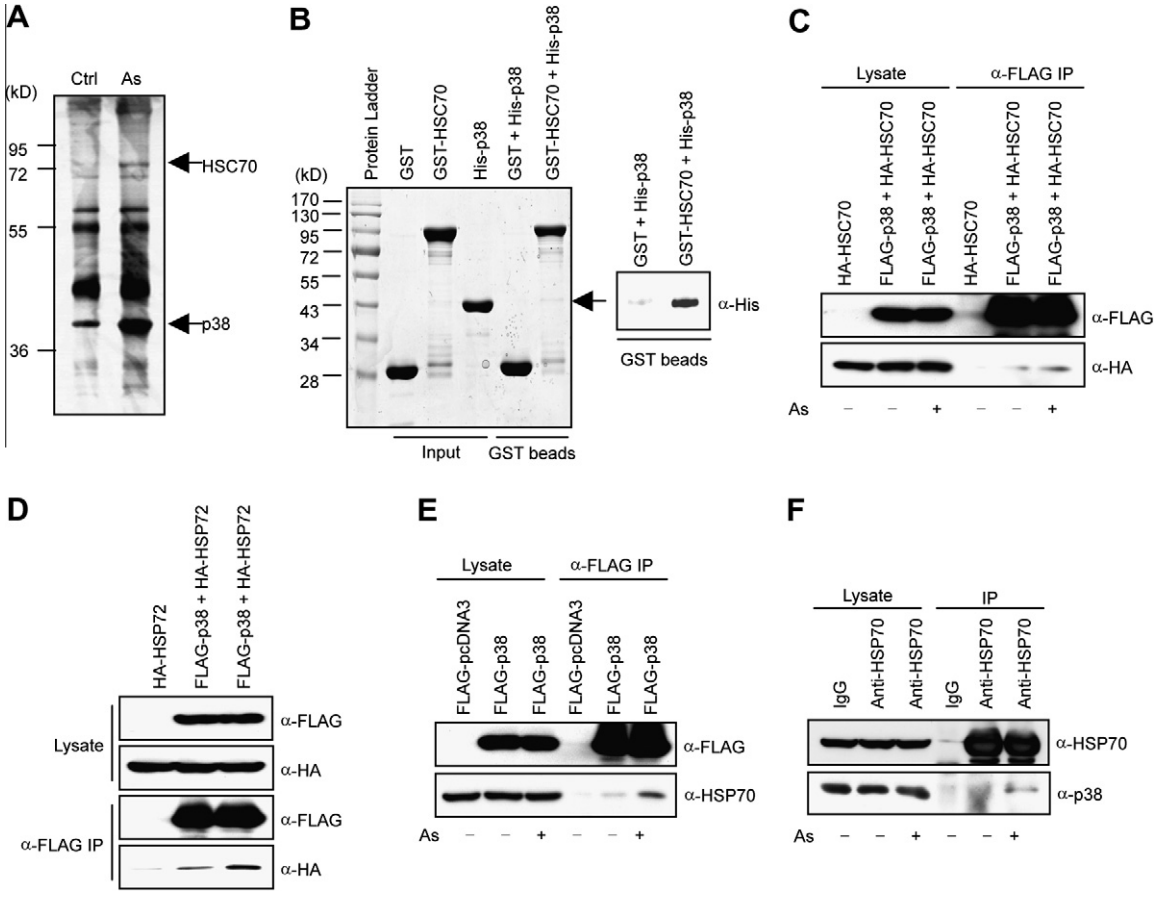

Fig. 1. Stress-induced interaction between p38 MAPK and HSP70. (A) HeLa cells were treated with or without 200 μM arsenite (As), lysed and incubated with a Sepharose beads-conjugated p-p38 antibody. (B) GST-tagged HSC70 and GST were purified and used for an *in vitro* binding assay with His-p38-bound Ni^{2+}-NTA Resin. (C and D) COS-7 cells were either transfected with HA-HSC70/HA-HSP72 alone, or co-transfected with FLAG-p38 and HA-HSC70/HA-HSP72 and stimulated with or without arsenite. Western blot analyses of whole cell extracts (WCE) and immunoprecipitates were performed using anti-FLAG or anti-HA antibodies. (E) COS-7 cells were either transfected with FLAG-pcDNA3 vector or FLAG-p38 and stimulated with or without arsenite. Western blot analyses of WCE and immunoprecipitates were performed using anti-FLAG or anti-HSP70 antibodies. (F) HeLa cells were stimulated with or without arsenite, then immunoprecipitation with HSP70 antibody was performed. Western blot analyses of WCE and immunoprecipitates were performed using anti-HSP70 or anti-p38 antibodies.

were seeded into 60 mm dishes. Twenty-four hours later, 3 μg DNA was mixed with 150 μl Opti-MEM (Invitrogen, USA), followed by further mixing with 15 μl Polyfect (Qiagen, Germany). The mixture was incubated at room temperature for 15 min before adding to the cells. After 24 h incubation in the cell incubator, the cells were treated and harvested. For siRNA transfection, 2×10^5 HeLa cells were seeded into 60 mm dishes. Twenty-four hours later, 200 pmol siRNA was transfected into HeLa cells with 20 μl Oligofectamine (Invitrogen, USA), with the instruction of the manufacturer's protocol. After 48 h further incubation in the cell incubator, the cells were treated and harvested.

2.5. Co-immunoprecipitation and immunoblotting

Cells were plated in 60 or 100 mm dishes and cultured to 80–90% confluence. The cells were washed with ice-cold PBS once, then lysed with 150 or 500 μl cell lysis buffer (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1 mM β-glycerophosphate, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 1 mg/ml leupeptin, and 1 mg/ml aprotinin) on ice for 20 min. Whole cell lysates were collected and centrifuged at 4 °C, 12,000 rpm for 10 min. The resultant supernatants were quantified with BCA protein quantification kit (Pierce/Thermo Scientific, USA). For co-immunoprecipitation with transfected cells from 60 mm dishes, 300 μg total proteins of each were used for incubation with anti-FLAG-coupled agarose beads for 3 h at 4 °C. For endogenous co-immunoprecipitation with anti-HSP70 antibody, 1 mg total proteins of each were used. After five washes with cell lysis buffer, the resultant immunopellets were subjected to 10% SDS–PAGE, followed by Western blot analysis with specific primary antibodies and corresponding HRP-labeled secondary antibodies, using an enhanced chemiluminescence detection system (Cell Signaling Technology, USA). Images were obtained with Kodak IS2000R image station (Kodak, USA).

2.6. Immunocytochemistry

HeLa cells were washed with Tris buffered saline (TBS) and fixed in 4% paraformaldehyde for 10 min. Washed once with TBS, the cells were permeabilized with 0.2% Triton X-100 for 5 min at room temperature. Washed three times, the cells were quenched in 0.1% $NaBH_4$ in TBS for 5 min. The cells were blocked by incubation for 1 h in TBS containing 3% BSA, and then incubated with p38 or HSP70 specific antibodies at 4 °C overnight. Washed three times with TBS and incubated with Alexa 488 or Alexa 594-conjugated secondary antibodies for 45 min at room temperature, the cells were then stained with 100 ng/ml DAPI to visualize nuclei. Images were obtained with the A4, L5 or N3 filters of DM-RA2 fluorescence microscope (Leica, Germany) equipped with a Leica DC100 digital camera.

3. Results

3.1. Pull-down assay indicated HSC70 a novel p38 MAPK-interacting protein

To search for stress-induced p38-interacting proteins, we performed an endogenous pull-down assay with a Sepharose beads-conjugated phospho-p38 antibody. As shown in Fig. 1A, there was a notable band with approximate molecular weight 72 kD was co-immunoprecipitated in arsenite-treated cells, as compared with untreated cells. It was identified as human HSC70 by mass spectrometry.

3.2. HSP70 interacted with p38 MAPK in vitro and in vivo

To confirm the result from mass spectrometry, we first performed an *in vitro* binding assay. GST-HSC70, but not GST, bound to p38, indicating that there is a direct interaction between

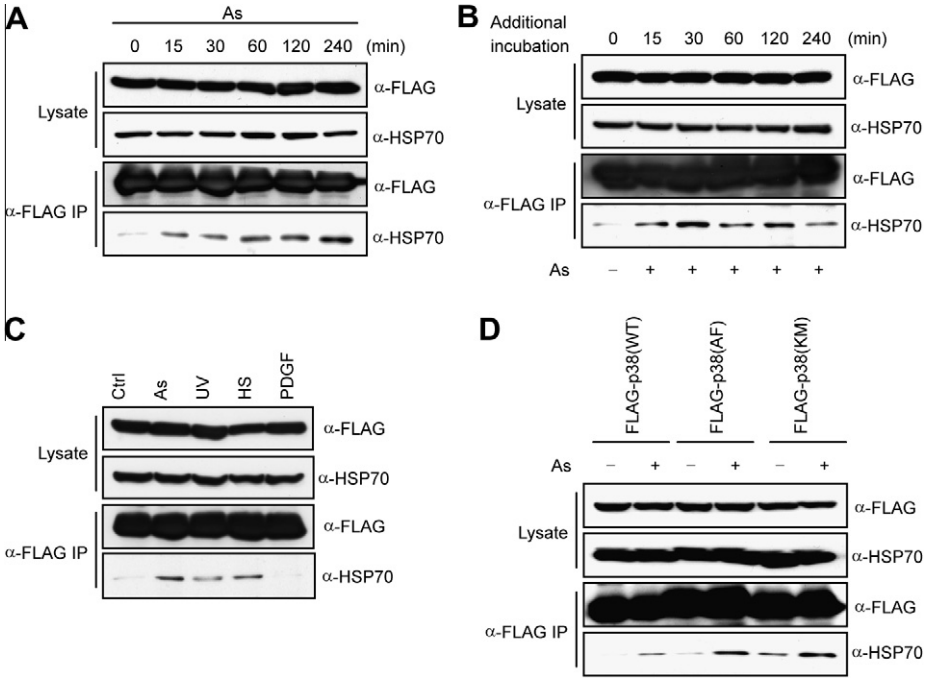

Fig. 2. Characterization of stress-induced interaction between HSP70 and p38 MAPK. (A) COS-7 cells were transfected with FLAG-p38, and stimulated with arsenite for the indicated times. (B) COS-7 cells were transfected with FLAG-p38, and stimulated with or without arsenite for 15 min, then further incubated for the indicated times. (C) COS-7 cells were transfected with FLAG-p38, and stimulated with or without arsenite, UV radiation (30 J/m^2), heat-shock (HS, 42 °C 30 min) or PDGF (100 ng/ml, serum starvation for 16 h before stimulation). (D) COS-7 cells were transfected with FLAG-p38(WT), FLAG-p38(AF), or FLAG-p38(KM), and stimulated with or without arsenite. Western blot analyses of WCE were performed using anti-FLAG or anti-HSP70 antibodies.

HSC70 and p38 (Fig. 1B). Arsenite-induced co-immunoprecipitation of FLAG-tagged p38 with HA-tagged HSC70 was confirmed (Fig. 1C). As HSP72 is the inducible isoform of HSP70, we also checked if HSP72 interacted with p38. As shown in Fig. 1D, with the treatment of arsenite, HA-tagged HSP72 was co-precipitated by FLAG-p38, too. We then used an antibody recognizing both HSC70 and HSP72, to investigate the interaction of p38 with the endogenous HSP70. Both FLAG-p38 and endogenous p38 interacted with HSP70 upon arsenite treatment (Fig. 1E and F), indicating that HSP70 interacts with p38 *in vivo*.

We then characterized the dynamic interaction between p38 and HSP70. As shown in Fig. 2A, when the cells were continuously treated with arsenite, we found that the binding activity between p38 and HSP70 kept increasing within 4 h. However, when the cells treated with arsenite for 15 min and then arsenite was washed out, their binding activity increased and reached to a peak within 30 min, then maintained at a relative high level in 2 h; 4 h after stimulation, the binding activity returned to the resting level (Fig. 2B).

We also wanted to know if the interaction between these two proteins was a common phenomenon in a cell's response to extracellular stimuli. As shown in Fig. 2C, among all different types of stimuli tested, including stresses (arsenite, ultraviolet (UV) radiation, and heat-shock) and growth factor (PDGF), only stresses induced the interaction, suggesting it is a stress-specific biological event.

The dual phosphorylation and kinase activity are crucial for p38 to perform its physiological functions [1]. Dominant negative mutant of p38, p38(AF), and kinase dead mutant, p38(KM), were employed to clarify if the interaction between HSP70 and p38 is p38 phosphorylation- or kinase activity-dependent [1,11]. As shown in Fig. 2D, none of them had blockade effect on the interaction, suggesting neither phosphorylation nor kinase activity of p38 is necessary for its binding to HSP70.

3.3. HSP70 interacted with p38 MAPK through its PBD and VD domains

HSC70 consists of three domains, the ATPase domain (AD), the protein binding domain (PBD), and the variable domain (VD) (Fig. 3A) [12]. To identify the interaction domain, the plasmids expressing different truncated forms of HSC70 were constructed and co-immunoprecipitation was performed. Both PBD and VD, but not AD of HSC70, were required for its interaction with p38 upon arsenite treatment (Fig. 3B), suggesting that the interaction is mediated by the PBD and VD domains together.

3.4. Stress-induced co-localization of p38 MAPK and HSP70

To study if there is stress-induced co-localization of p38 and HSP70, their intracellular localizations were investigated by immunofluorescence with corresponding specific antibodies. As shown in Fig. 4, in untreated cells, p38 and HSP70 both dispersed in the whole cell. With the treatment of stresses, including arsenite, UV radiation, and heat-shock, both of these two proteins translocated into the nuclei, showing a prominent co-localization. Meanwhile, no significant localization change was found in PDGF-treated cells. These results further confirmed the findings in Fig. 2C.

3.5. HSP70 may play a role in p38 MAPK's phosphorylation to its nuclear substrate, MK2

To explore the potential physiological functions of HSP70 through its interaction with p38, siRNAs targeting HSC70 were employed to study its effects on the phosphorylation of a specific downstream substrate of p38, MK2 [1,2,11,13]. Consistent to others' reports, we found that specific knockdown of HSC70 greatly

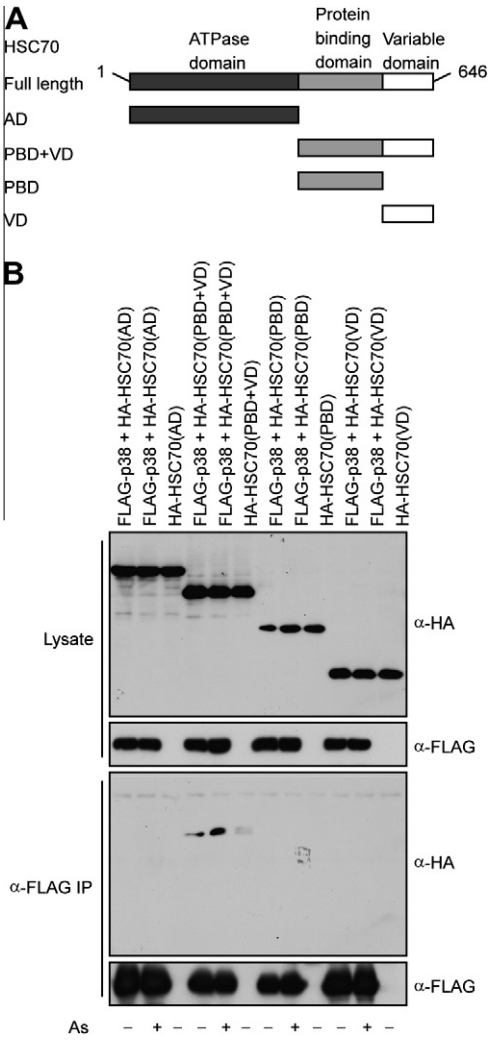

Fig. 3. Mapping of the interacting domains of HSC70. (A) Stretch of HSC70 domains. (B) COS-7 cells were co-transfected with FLAG-p38 and HA-tagged full-length or different truncated forms of HSC70. Western blot analyses of WCE and immunoprecipitates were performed using anti-FLAG or anti-HA antibodies.

enhanced the expression of HSP72, which made the total amount of HSP70 significantly increased [14–16]. The phosphorylation of p38 itself was not affected by the increased expression level of HSP70, while that of MK2 was notably enhanced (Supplementary Fig. 1). This result suggested that the interaction of HSP70 with p38 may play a role in p38's phosphorylation to its downstream substrates in the nucleus.

4. Discussion

The HSP70 family proteins function as molecular chaperones, including at least eight members with diverse biochemical features [17–20]. They are either expressed constitutively or induced in response to various types of stress, including heat-shock, ischemia, oxidative stress, and exposure to toxins [15–17,20,21]. Two major cytoplasmic isoforms are constitutive form HSC70 (heat-shock cognate 70) and inducible form HSP72 (heat-shock protein 72), which show 86% sequence identity [14,17,22]. HSC70 is abundantly and ubiquitously expressed, while HSP72 expresses relatively low in the absence of stress and its expression is significantly induced via heat-shock factor 1 (HSF1) activation upon stresses [17,20]. They function in ensuring proper nascent

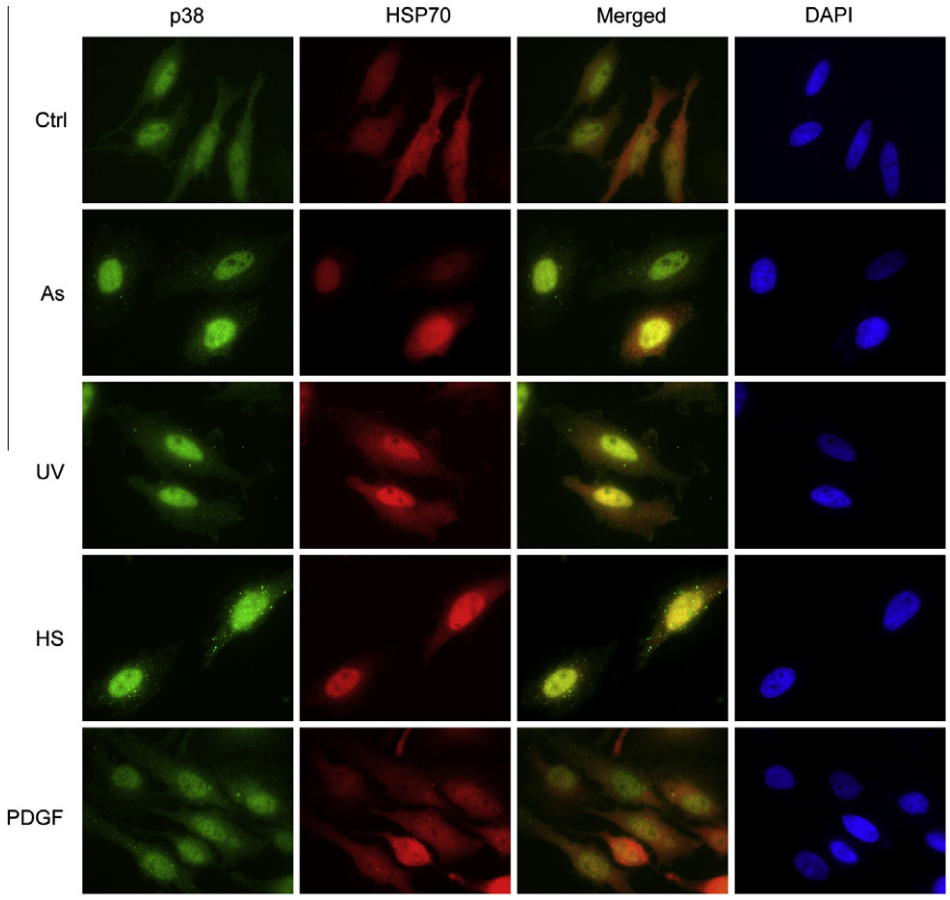

Fig. 4. Stress-induced co-localization of p38 MAPK and HSP70. HeLa cells were stimulated with or without arsenite, UV radiation, heat-shock, or PDGF, and double-stained with primary antibodies against p38 or HSP70, followed by incubation with Alexa Fluor 488 or 594-conjugated secondary antibodies, respectively. The nuclei of cells were stained with DAPI.

protein folding, preventing denatured protein aggregation, assisting in protein trafficking between cellular compartments such as endoplasmic reticulum and nucleus, and modulating assembly/disassembly of protein complexes [17,20,21,23–26].

HSC70 shuttles between the cytoplasm and the nucleus, trafficking cargo proteins to the nucleus after the cell is exposed to stresses [27,28]. Several mechanisms of HSC70 nuclear import have been proposed. HSC70 itself contains a functional nuclear localization signal (NLS), a nuclear localization-related signal (NLRS), and a nuclear export signal (NES), and its nuclear localization depends on the co-import of cargo [12]. Both HSC70 and HSP72 promote the formation of NLS-cargo-importin-α complex, regulating the nuclear import of cargo proteins, such as NF-κB, mutated p53, and nucleoplasmin [19,28]. In addition, HSC70 may broadly regulate nucleocytoplasmic transport systems via its role in the nuclear export of importins and transportin [27,28].

Endogenous p38 is distributed both in the cytosol and nucleus in resting cells, and cytosolic p38 translocates into the nucleus upon activation to access its nuclear substrates [1,11]. With the disappearance of the stimulation, inactivated p38 is exported to the cytosol to receive the next stimulation. Our previous study showed LPS stimulation promptly induced the nuclear translocation of p38 to mediate the upregulation of tumor necrosis factor α (TNF-α) expression in monocytes, highlighting the importance of the localization of p38 on its biological function [11]. Thus, stimuli-induced intracellular redistribution is an important functional aspect of p38. The nuclear translocation of p38 depends on its phosphorylation but not its catalytic activity [11]. In budding yeast, the nuclear translocation of p38 is a NLS-dependent active process. As no typical NLS could be identified in p38, and no direct interactions have been detected between p38 and purified importins, it may undergo nuclear translocation through interaction with other protein with a NLS as a carrier.

Although in most cases, HSC70 and HSP72 are regarded as equivalent and functionally interchangeable, it is suggested that HSC70 and HSP72 substitute for each other in healthy cells, whereas HSP72 is essential for certain cells to respond to cytotoxic factors [16,17,29,30]. In our case, they seem to have similar functions based on their interactions with p38. As shown in Supplementary Fig. 1, HSP70 may play a role in the phosphorylation of MK2 by p38. Given that MK2 locates in the nucleus in resting cells and translocates to the cytosol upon stress-induced phosphorylation by p38, its phosphorylation should take place in the nucleus. In our previous study, we proposed a model in which p38 translocated into the nucleus along the microtubule, with the help of a NLS-containing protein, as well as the assistance of dynein [11,31]. Based on our findings in this study, we propose that HSP70 may be this exact NLS-containing protein, which helps p38 to access its nuclear substrates [21,26]. This presumption is supported by their stresses-induced interaction and co-localization. There is also a report about the functional relationship between dynein and HSP70, in which the interaction between Bcl-2-associated athanogene 3 and dynein selectively directs HSP70 substrates to the motor [32].

It is suggested that HSC70 is a possibly more dominant form, as HSC70 is involved in HSP72 expression, but not vice versa [17,19].

In addition, as stress-induced nuclear translocation of p38 is fairly rapid, the physiological player mainly involved in this process should be HSC70. We hence knocked-down HSC70 to explore its effect on the translocation of p38. However, as we and others have shown, knockdown of HSC70 greatly enhanced the expression of HSP72, leading to the total amounts of HSP70 considerably increased. Although our loss-of-function study actually turned into a gain-of-function one, it somehow suggested the potential effect of HSP70 in p38 translocation.

The phosphorylation of p38, but not its kinase activity, plays a key role in determining its intracellular localization [7]. However, either p38(AF) or p38(KM) bound to HSP70 more strongly than wild-type p38 did, suggesting a probable feedback in this process. Moreover, as p38(AF) interacted to HSP70 but failed to translocate, there must be some other mechanisms involved, too.

Future studies may focus on the confirmation and mechanism studies on the effects of HSP70 on p38 translocation, by means of simultaneous knockdown of HSC70 and HSP72, expression of truncate forms of HSC70 and mutants of HSC70 NLS or ATP binding site, etc. The fulfilment of these studies may provide us an overall knowledge about the mechanisms for the stress-induced nuclear translocation of p38.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.07.096.

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