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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

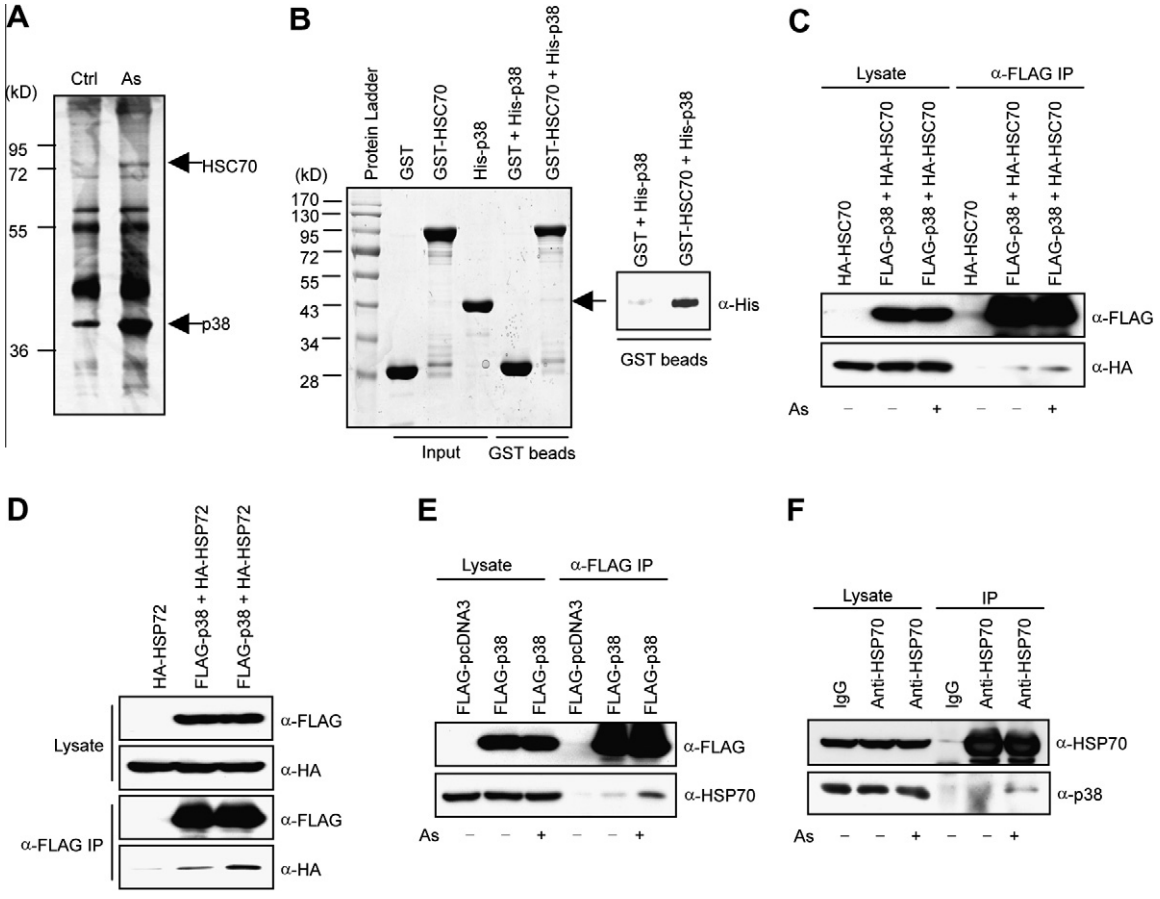

Fig. 1. Stress-induced interaction between p38 MAPK and HSP70. (A) HeLa cells were treated with or without 200 μM arsenite (As), lysed and incubated with a Sepharose beads-conjugated p-p38 antibody. (B) GST-tagged HSC70 and GST were purified and used for an *in vitro* binding assay with His-p38-bound 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.