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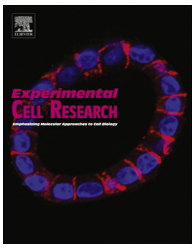

Research Article

Nmi interacts with Hsp105β and enhances the Hsp105β-mediated Hsp70 expression

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ABSTRACT

The mammalian stress protein Hsp105α is expressed constitutively and is further induced under stress conditions, whereas the alternative spliced form, Hsp105β is only expressed during mild heat shock. We previously reported that Hsp105α is localized mainly in the cytoplasm, whereas Hsp105β is localized in the nucleus. Consistent with the different localization of these proteins, Hsp105β but not Hsp105α induces the expression of the major stress protein Hsp70. We here identified N-myc and Stat interactor (Nmi), as an Hsp105β-binding protein by yeast two-hybrid screening. Immunoprecipitation and pull-down assay showed that Nmi interacts with Hsp105β *in vivo* and *in vitro*. Luciferase reporter gene assay and Western blotting showed that Nmi enhanced both the Hsp105β-induced phosphorylation of Stat3 and the Hsp105β-induced activation of the *hsp70* promoter in a manner that is dependent on the Stat3-binding site, which results in an increase in Hsp70 protein levels. Most importantly, mild heat shock-induced Hsp70 expression, which is dependent on Hsp105β, is suppressed by knockdown of endogenous Nmi. These results suggest that Nmi has a role as a positive regulator of Hsp105β-mediated *hsp70* gene expression along the Stat3 signaling pathway.

Introduction

Heat shock proteins (Hsps) are constitutively expressed and function as molecular chaperones. The most characterized Hsps, Hsp70 family proteins, have been shown to facilitate the proper folding and prevent misfolding of newly synthesized polypeptides under normal conditions [1–3]. Hsp70 expression is further induced under stress conditions such as heat shock, and Hsp70 subsequently prevents the irreversible aggregation of denatured proteins [4]. Hsp70 modulates apoptosis by inhibiting c-Jun N-terminal kinase (JNK), apoptosis signal-regulating kinase 1 (ASK1), and caspases [5], and also modulates important cellular functions such as cellular senescence and autophagy [6–8]. These findings indicate that Hsp70 protects cells from cell death under stress conditions in multifunctional ways.

The induction of Hsp70 expression in mammalian cells is controlled by heat shock factor 1 (HSF1), a master regulator of the heat-inducible expression of *hsp* genes. HSF1 is constitutively

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expressed in an inactive conformation under non-stressed conditions. When cells are exposed to stress, HSF1 is trimerized, translocated to the nucleus, and bound to the heat shock element (HSE), which activates the expression of Hsp70 transcription [9]. When Hsp70 accumulates in cells, it interacts with the carboxy-terminal activation domain of HSF1 and inhibits the binding of HSF1 to HSE [10]. Thus, HSF1 transcriptional activity is negatively regulated by feedback from the Hsp70 expression levels.

The expression of Hsp70 is also controlled by several transcription factors. The c-Myc responsive element for upregulation is detected in *Drosophila* and human *hsp70* promoters [11,12]. NF-Y, a CCAAT box-binding protein, binds the CCAAT sequence of the *hsp70* promoter and enhances the cell cycle-specific induction of Hsp70 cooperatively with c-myc [13]. GATA-1 is a hematopoietic transcription factor that induces Hsp70 in oncogenic tyrosine kinase BCR/ABL-expressing cells [14]. Stat1 mediates the cytokine-induced expression of Hsp70 [15]. Hsp70 expression is upregulated by other heat shock proteins, such as Hsp105β, through the Stat signaling pathway [16], which suggests the existence of a chaperone network between these proteins.

The Hsp105/110 family members, Hsp105α and Hsp105β are alternatively spliced products derived from an *hsp105* gene transcript, and Hsp105β lacks 44 amino acids from Hsp105α [17]. Hsp105α is expressed constitutively and is further induced by various stresses, whereas Hsp105β is specifically expressed during mild heat shock [18–21]. These proteins suppress the aggregation of proteins denatured by heat shock, as does Hsp70 [22,23]. Furthermore, Hsp105α and Hsp105β form complexes with either Hsp70 or Hsc70, and regulate the Hsp70 chaperone system [24,25]. We previously reported that Hsp105α and Hsp105β suppress stress-induced apoptosis including oxidative stress, which indicates their important role in the protection of cells against stress-induced apoptosis [26,27]. Hsp105β is localized to the nucleus, whereas Hsp105α is localized in the cytoplasm [28]; therefore, Hsp105β may have different functions from those of Hsp105α. We recently showed that Hsp105β, but not Hsp105α induces the expression of Hsp70 [29], suggesting that Hsp105β may play an important cooperative role in the protection of cells with Hsp70 under heat shock conditions. However, the precise mechanism by which the Hsp105β-mediated Hsp70 expression is induced has not yet been elucidated. In the present study, we attempted to identify the proteins that bind to Hsp105β and modulate the expression of Hsp70.

We have shown here that N-myc interactor (Nmi), which is known as a Stat-binding protein, interacts with Hsp105β and enhances Hsp105β-mediated *hsp70* promoter activity in a manner that depends on the Stat3-binding site, resulting in an increase in Hsp70 protein levels. Our results suggest that Nmi may act as a transcription activator through the Stat signaling pathway in order to induce the expression of Hsp70.

Materials and methods

Plasmids

The yeast two-hybrid bait plasmid pGBK105β, which expressed the fusion protein of the GAL4-DNA binding domain and Hsp105β, was constructed as follows. The full-length of the gene encoding Hsp105β was amplified from pTrc105-2 plasmid DNA [22] by PCR using both sense (5′–GGGGTACCAGCCATGTCGGTGGTGGTT–3′) and antisense (5′–AATCTTCTCTCATCCGCC–3′) primers. The amplified DNA fragment was digested with *Kpn*I and cloned into the *Kpn*I site of the pHybLex/Zeo vector (Invitrogen). The resulting plasmid was digested with *EcoR*I and *Sal*I and cloned into the same sites of the pGBK-T7 vector (Clontech). To construct the expression plasmid for Flag-tagged Nmi (pcDNA–Flag–Nmi), full-length Nmi cDNA was amplified from the total RNA of HeLa cells by RT-PCR using both sense (5′–GGGCTCG-AGCTATTCTTCAAAGTATGCTATG–3′) and antisense (5′–CGGGATCCC-GGGGGATCATGGAAGC–3′) primers. The amplified DNA fragment was digested with *BamH*I and *Xho*I, and then cloned into the same sites of the pcDNA-Flag vector [30]. The mammalian expression plasmids for mouse Hsp105α (pcDNA105α) and Hsp105β (pcDNA105β) have been previously described [28]. The luciferase reporter plasmids [pGL70 (-2616), pGL70(−218), pGL70(−218)m], in which the promoter regions of the *hsp70* gene were inserted upstream of the *luciferase* gene, have been previously described [16].

Yeast two-hybrid screening

Yeast two-hybrid screening was performed with Matchmaker two-hybrid system 3 (Clontech) and the Pretransformed Normalized Matchmaker Human HeLa S3 Library (Clontech). In brief, the yeast strain, AH109, which was transformed with the bait plasmid pGBK105β, was mated with the Y187 strain, which had been pretransformed with the HeLa cDNA library, to prepare diploid cells. Positive clones were screened for the expression of the reporter genes (*ADE2*, *HIS3*, and *MEL1*) according to the manufacturer's instructions (Clontech). Prey plasmids were isolated from positive clones. Yeast was transformed with the prey plasmid and mated with the bait strain, pGBK105β/AH109, to confirm the two-hybrid interaction.

Cells and transfection

African monkey kidney COS-7 cells and human epithelial carcinoma HeLa cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum under a 5% CO_2 atmosphere at 37 °C. Transfection was performed using DMRIE-C reagent (Invitrogen) according to the manufacturer's instructions.

Antibodies

Mouse monoclonal anti-Flag (clone M2, Sigma), anti-Nmi (clone 9E8, Abnova), anti-Hsp70 (clone C92F3A-5, Enzo Life Sciences), anti-α-tubulin (clone DM1A, Sigma) and anti-actin (clone AC-40, Sigma) antibodies were used. Rabbit polyclonal anti-mouse Hsp105 antibody, which only reacts with mouse Hsp105 [30], anti-human Hsp105 antibody, which reacts with human, mouse and monkey Hsp105 [21], anti-Stat3 (K-15, Santa Cruz Biochemistry) and anti-phospho-Stat3 (Tyr705) (D3A7, Cell Signaling Technology) were used. Horseradish peroxidase-conjugated anti-mouse IgG and anti-rabbit IgG antibodies (Santa Cruz Biotechnology) were also used.

Western blotting

Cells were lysed with 0.1% SDS, subjected to SDS-PAGE, and then transferred onto nitrocellulose or PVDF membranes. To examine the phosphorylation status of Stat3, cells were lysed with SDS-sample buffer containing phosphatase inhibitors (10 mM Na_3VO_4, 20 mM β-glycerophosphate and 50 mM NaF) and protease inhibitors (2 μg/ml aprotinin, 0.8 μg/ml pepstatin A, 2 μg/ml leupeptin and 2 mM PMSF). Proteins were sequentially incubated with the appropriate primary