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Activation of HRI is mediated by Hsp90 during stress through modulation of the HRI-Hsp90 complex

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ABSTRACT

Heme Regulated Inhibitor (HRI) is known to get activated in various stresses such as heme deficiency, heat shock, heavy metal toxicity *etc*. Heat shock protein 90 (Hsp90), a ubiquitous cytoplasmic protein interacts with HRI in order to regulate protein synthesis. However, it still remains to establish this interaction of HRI and Hsp90 at cellular levels and how this modulation of HRI activity is mediated by Hsp90 during stress. In the present report, using co-immunoprecipitation analysis we show that HRI interacts with Hsp90 and this association is independent of other co-chaperones in *in vitro* conditions. Further, analysis using truncated domains of HRI revealed that the K1 subdomain is essential for HRI - Hsp90 complex formation. Our *in silico* protein - protein interaction studies also indicated interaction of Hsp90 with K1 subdomain of HRI. Mammalian two hybrid assay validated this HRI - Hsp90 interaction at cellular levels. When the *in vitro* kinase assay was carried out with the co-immunoprecipitated complex of HRI - Hsp90, an increase in the kinase activity was observed resulting elevated levels of eIF2α phosphorylation upon heavy metal stress and heat shock. Thus, our results clearly indicate modulation of HRI kinase activity with simultaneous Hsp90 association under stress conditions.

1. Introduction

Eukaryotic initiation factor 2 (eIF2) regulates eukaryotic initiation of translation through its phosphorylation/dephosphorylation and thus plays a crucial role in the regulation of protein synthesis in eukaryotic cells [1, 2]. The phosphorylation of eIF2α (Ser51) by eIF2α kinases is one of the key mechanisms of translational control during stress and is an early event associated with the down - regulation of protein synthesis at the level of translation initiation [3]. There are four known mammalian eIF2α kinases that phosphorylate eIF2α at Ser51 under different conditions as they are regulated by mechanisms unique to their own [4]. The four eIF2α kinases, classified on the basis of the mechanism of their activation, are as follows: Eukaryotic Initiation Factor 2 alpha Kinase 1 (EIF2AK1), also known as Heme Regulated Inhibitor (HRI), which gets activated by heme-deficiency, oxidative stress, heavy metal stress, *etc*. [5]. EIF2AK2, also known as Protein Kinase, dsRNA dependent (PKR), activated by viral infection, IFNɣ, *etc*. [6]. EIF2AK3, also known as PKR - like Endoplasmic Reticulum Kinase (PERK) or Pancreatic eIF2α kinase (PEK), activated by misfolded proteins in the ER [7]. EIF2AK4, also known as General Control Non - derepressible 2 Kinase (GCN2), activated by amino acid deficiency and UV - irradiation [8].

Heat shock proteins such as Hsp90 and Hsp70, are ubiquitously expressed molecular chaperones that facilitate protein folding, regulate quality control and guide protein turnover in an effort to maintain cellular homeostasis [9]. Hsp90 is one of the most abundant cytosolic proteins in various eukaryotic cells at normal temperatures. The level of Hsp90 is increased upon heat shock, underscoring its importance in helping cell survival under such conditions [10]. In eukaryotes, constitutive genetic knockout of Hsp90 is lethal. Hsp90 resides primarily in the cytoplasm, where it exists predominantly as a homo - dimer [11]. Dimerization of Hsp90 is required to position the catalytic machinery for efficient ATP hydrolysis [12]. Hsp90 controls the biogenesis, stability and activity of a specific and discrete set of client proteins, particularly protein kinases [13–15]. It is recruited to its kinase clients through interactions with co-chaperones, such as Cdc37 that link Hsp90 and the kinase client [16, 17]. This mechanism is revealed in a structural analysis of the Cdc37 - Cdk4 - Hsp90 complex [18]. Several studies have reported that a chaperone recognizes a common surface in amino terminal lobe of kinases from diverse families than a contiguous amino acid sequence [13, 15, 19, 20].

eIF2α kinases have been reported to associate and interact with Hsp90 during their maturation and/or activation. Hsp90 forms a

Abbreviations: HRI, Heme regulated inhibitor; eIF2α, Eukaryotic initiation factor 2 alpha; Hsp90, Heat shock protein 90; Cdc37, Cell division Cycle 37; iRDP, *in silico* Rational Design of Proteins; M2H, Mammalian two-hybrid.

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complex with GCN2 *in vitro* as well as *in vivo*. GCN2 requires the molecular chaperone Hsp90 for proper regulation. Immature GCN2 binds more tightly to Hsp90 and that GCN2 forms a complex with Hsp90 during GCN2 synthesis [21]. Similarly, PKR associates with Hsp90 - p23 complex during maturation; this interaction is terminated by geldanamycin (GA), an inhibitor of Hsp90 [22, 23]. PERK also associates with Hsp90 and GA disrupts this association; but, PERK activity is much less dependent on Hsp90 in comparison to GCN2 or PKR [24].

HRI and Hsp90 interactions have been reported previously from our laboratory and several other groups [19, 25–28]. In one of the reports the domain structure of HRI was dissected to identify the segments which interact with Hsp90 [19]. This study reported that Hsp90 and Cdc37 recognize motifs present in N - terminal domain of catalytic kinase domain of HRI. Further, HRI - Hsp90 interactions are also reported for maturation of the kinase during biogenesis and this interaction is also suggested to be involved in activation of the kinase under conditions of stress particularly during heat-shock [25–28]. However, it still remains to establish this interaction at cellular levels and also how this modulation of HRI activity is mediated by Hsp90 during stress, particularly during heat shock. Therefore, in the present investigation we have studied the interaction of HRI - Hsp90 *in vitro* by co-immunoprecipitation and by performing mammalian two-hybrid assay and *in silico* by protein - protein interactions. The current study highlights the direct association of HRI - Hsp90 without an aid of co-chaperone. We report an enhancement in HRI activity in terms of elevated levels of eIF2α phosphorylation upon interaction with Hsp90 under stress conditions such as heat shock and heavy metal stress.

2. Materials and methods

2.1. Materials

Mammalian cell lines (K562 and HeLa) were obtained from the cell repository, National Centre for Cell Science, Pune, India. All the cell culture reagents, namely, Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), antibiotic - antimycotic solution (100×), β-actin antibody, secondary antibodies (Goat anti - rabbit and anti - mouse IgG conjugated to HRP) and most of the molecular biology reagents were purchased from Sigma Chemical Co. (USA). Competent cells (BL21 Rosetta) and pET28a vector were obtained from Novagen, Germany. The primary antibodies (anti - HRI, anti - Hsp90 and anti - phospho eIF2α-Ser51) were purchased from Abcam Plc., UK. Anti - His antibody was obtained from Santa Cruz Biotechnology, USA.

2.2. Plasmid constructs and protein expression

Total RNA was isolated from K562 cells using TRIzol reagent as per manufacturer's instructions. The cDNA was prepared after DNase digestion of the RNA sample and was used as the template for PCR amplification of HRI, eIF2α and Hsp90 using oligos as mentioned in Table 1. The PCR amplified fragments were cloned in pET28a vector followed by transformation in *E. coli* (DH5α strain). Positive clones were screened by restriction digestion and DNA sequencing. For overexpression of recombinant polypeptides, the cloned plasmids were transformed in *E. coli* BL21 Rosetta cells. The protein was expressed using IPTG induction at the final conc. of 1 mM. Cells were harvested 4 h post induction and centrifuged at 8500*g* for 30 min. The cell pellet (biomass) was stored at −80 °C till further use.

Table 1
List of Primers.

Sr no.	5′ → 3′ Sequence (Restriction site is underlined)	
1.	HRI-FP	GGCAATTCCATATGCAGGGGGGCAACTCCGG
2.	HRI-RP	CCTTGCTCGAGTCCCACGCCCCCATCCTTTCC
3.	HRI-M2H-FP	TCATACGCGTTGCAGGGGGGCAAC
4.	HRI-M2H-RP	GTAGGTACCTCCCACGCCCCCATC
5.	eIF2α-FP	GGCAATTCCATATGCCGGGTCTAAGTTGTAG
6.	eIF2α-RP	CCTTGCTCGAGATCTTCAGCTTTGGCTTCC
7.	Hsp90-FP	GCTTCCCATGGCTGAGGAAACCCAGAC
8.	Hsp90-RP	CCTTGCTCGAGGTCTACTTCTTCCATGCGTG
9.	Hsp90-M2H-F2	CTTAGCTCACGCGTTGCCTGAGGAAACCCAGACC
10.	Hsp90-M2H-R2	TCATCTCAGGTACCGTCTACTTCTTCCATGCGTG

2.3. Purification and refolding of polypeptides

Recombinant polypeptides - HRI (full length), truncated domains of HRI (ΔCTD lacks *C-terminal* domain; ΔNTD lacks *N-terminal* domain; K2 is *kinase 2* subdomain; N stands for *N-terminal* domain; K1 is *kinase 1* subdomain; and, I denotes *kinase insert* region) (Fig. 1), eIF2α and Hsp90 were overexpressed in *E. coli* and the inclusion bodies (IBs) were isolated using the solubility analysis protocol mentioned previously [29]. The IBs were solubilized in 8 M Urea with 50 mM Tris - HCl (pH 10.0) and 100 mM glycine and concentrated using Amicon ultrafiltration column (Millipore). The solubilized IBs were then buffer exchanged 3 times with 6 M Urea (pH 3.4) and then sample was concentrated back. The concentrated sample was then refolded using rapid dilution method as described previously [29]. Sample was added to refolding buffer (L-arginine, glutathione reduced, glutathione oxidized, ethanolamine; pH 8.0) at the final concentration of 50 μg/ml. The refolded protein was concentrated and then dialyzed against 10 mM phosphate buffered saline (PBS). The dialyzed protein was centrifuged at 10,000*g* for 30 min and the supernatant was filtered through 0.2 μ syringe filter and stored in aliquots at −80 °C till further use.

2.4. In vitro cell culture and stress challenge to cells

Human K562 and HeLa cells were maintained as continuous culture in DMEM supplemented with 10% FBS at 37 °C and 5% CO_2 with antibiotic - antimycotic solution. To generate stress, cells were exposed to two different cytoplasmic stresses *viz.* heavy metal exposure (lead acetate 100 μg/ml for 8 h) and heat - shock (42 °C, 1 h).

2.5. Protein extraction and SDS PAGE

Protein extraction from control and treated cells was done using protein extraction buffer [20 mM Tris - HCl (pH 8.0), 1 mM EDTA, 0.1% Triton X-100, 1 mM phenylmethylsulphonylfluoride (PMSF)] and protease inhibitor cocktail (Roche). The supernatant containing soluble protein was obtained by centrifugation at 13,000 rpm at 4 °C for 30 min. The proteins were quantified by Bradford's assay [30] and equal quantities of protein were then separated by SDS PAGE [31].

2.6. In vitro kinase assay and western blot analysis

In vitro kinase assay was carried out as described previously [32]. Briefly, the kinase reaction mixture containing kinase buffer [20 mM Tris - HCl (pH 7.6), 2 mM magnesium acetate, 40 mM KCl], 0.5 mM ATP, purified human eIF2α and HRI was incubated at 37 °C for 30 min. After incubation, the reaction was terminated by the addition of Laemmli sample buffer [31] and then heated for 5 min at 95 °C and subjected to SDS PAGE. Proteins were electrophoretically transferred onto nitrocellulose membranes [33]. The blots were then processed for immunoreactions using appropriate antibodies. In brief, the blots were saturated with 1× blocking reagent (Roche) in Tris - buffered saline containing 0.1% *v*/v Tween - 20 (TBST, pH 7.5) for 1 h and incubated first with primary antibody (anti - phospho - eIF2α and anti - eIF2α) for 1 h at RT or overnight at 4 °C and then with secondary antibody (HRP - conjugated) for 1 h at RT with TBST washes (3 × 5 min) in between. The blots were developed using the chemiluminescence detection kit (Roche) and the results were analysed using Bio - Rad gel documentation system.

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