Chapter 5

Results

Studies on interaction of EIF2AK1 and HSP90
Interaction studies of EIF2AK1 and HSP90

We have discussed in detail in chapter 1 that EIF2AK1 is known to interact with HSP90 for its maturation during biogenesis and this interaction is also suggested to be involved in activation of the kinase under conditions of stress particularly during heat-shock (Matts et al., 1992; Pal et al., 1996, 1998; Xu et al., 1997). Previous studies and recent report from our lab have also emphasized on this role of HSP90 as an activator of EIF2AK1 (discussed in detail in Introduction).

It still remained to establish this interaction in vivo and also how this modulation of EIF2AK1 activity is mediated by HSP90 during stress, particularly during heat-shock. Therefore one of the objectives of this thesis was to determine the interaction of EIF2AK1-HSP90 in vivo and the possible mechanism involved in activation of the kinase. This chapter deals with the in vitro studies (co-immunoprecipitation) done with the purified EIF2AK1 polypeptides (full-length and different domains) and HSP90 and in vivo experiments for Mammalian two-hybrid assay.

Co-immunoprecipitation of HSP90 and EIF2AK1 polypeptides

Briefly, the co-immunoprecipitation of HSP90 and EIF2AK1 polypeptides was done by pre-incubation of the two at room temperature after which anti-HSP90 monoclonal antibody (MAb) was added and the reaction mix incubated at 4 °C overnight. The immune-complex thus formed was isolated by Protein A/G agarose beads and analysed by SDS-
The protocol is schematically represented in Fig. 5.1. The EIF2AK1 polypeptide was detected with anti-His-tag antibody while HSP90 was probed with rabbit anti-HSP90 polyclonal antibody (PAb). Anti-β-actin antibody was used for non-specific control as an antibody that did not bind to either HSP90 or EIF2AK1 and shall thus not pull either of them in the final eluate.

Fig. 5.1. Schematic representation of co-immunoprecipitation of HSP90 and EIF2AK1 (and its different domains).

The purified full-length EIF2AK1 was incubated with HSP90 under \textit{in vitro} conditions and the complex formed was immunoprecipitated with anti-HSP90 antibody (Fig. 5.2). The upper panel illustrates the EIF2AK1 polypeptide band which co-immunoprecipitated with the HSP90 that indicated direct association of the two proteins \textit{in vitro}.

This result also suggested that the two proteins could interact with each other even in absence of other proteins under these conditions; this
association is sufficient for the activation of EIF2AK1 in this complex during heat-shock as was demonstrated by Pal et al. (1998).

**Fig. 5.2. Full-length EIF2AK1 forms a complex with purified HSP90.** Lane 1 indicates the input level of the two proteins EIF2AK1 and HSP90; Mouse IgG was added as negative control to lane 2; the immunoprecipitate obtained with anti-HSP90 antibody was loaded in lane 3; and lane 4 contained immunoprecipitate obtained with anti-β-actin (ACTB) antibody (non-specific for both EIF2AK1 and HSP90).

The polypeptide ΔCTD (lacked the C-terminus of the full-length EIF2AK1) was also found to be associated with purified HSP90 under *in vitro* conditions and it co-immunoprecipitated with anti-HSP90 antibody (Fig. 5.3). The two polypeptides interacted with each other as is evident from the upper panel which shows the ΔCTD probed with anti-His-tag antibody. This indicates that the contribution of the C-terminal domain of EIF2AK1 in its association with HSP90 may either be negative or limited.
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Fig. 5.3. ΔCTD forms a complex with purified HSP90. Lane 1 indicates the input level of the two proteins ΔCTD and HSP90; Mouse IgG was added as negative control to lane 2; the immunoprecipitate obtained with anti-HSP90 antibody was loaded in lane 3; and lane 4 contained immunoprecipitate obtained with anti-β-actin (ACTB) antibody (non-specific for both ΔCTD and HSP90).

As the C-terminal domain consists of ~30 amino acids, there is less possibility of the same playing any crucial role in the association of EIF2AK1 with HSP90 and thereby its activation. The ΔCTD is functionally very similar to the full-length EIF2AK1 and that it also co-immunoprecipitates with HSP90 indicates towards other domains being more important for HSP90 binding than the C-terminal domain.

The polypeptide ΔNTD (lacked the N-terminal domain of EIF2AK1) was also associated with purified HSP90 under in vitro conditions and it co-immunoprecipitated with anti-HSP90 antibody (Fig. 5.4). This indicated that the N-terminal domain was not obligatory for the EIF2AK1-HSP90 interaction and hinted that the remaining domains contributed more to the association of EIF2AK1 with HSP90.
Fig. 5.4. ΔNTD forms a complex with purified HSP90. Lane 1 indicates the input level of the two proteins ΔNTD and HSP90; Mouse IgG was added as negative control to lane 2; the immunoprecipitate obtained with anti-HSP90 antibody was loaded in lane 3; and lane 4 contained immunoprecipitate obtained with anti-β-actin (ACTB) antibody (non-specific for both ΔNTD and HSP90).

The K1IK2 polypeptide (lacked both the N-terminal and C-terminal domains) also associated with purified HSP90 and co-immunoprecipitated with anti-HSP90 antibody (Fig. 5.5). K1IK2 consisted of the minimum regions sufficient for phosphorylation of the substrate eIF2α. Hence this polypeptide was conformationally and functionally self-sufficient, and its binding with HSP90 suggests that one of these three domains (kinase 1, kinase insert and kinase 2 domains) is necessary for the interaction of EIF2AK1 with HSP90.
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Fig. 5.5. K1IK2 forms a complex with purified HSP90. Lane 1 indicates the input level of the two proteins K1IK2 and HSP90; Mouse IgG was added as negative control to lane 2; the immunoprecipitate obtained with anti-HSP90 antibody was loaded in lane 3; and lane 4 contained immunoprecipitate obtained with anti-β-actin (ACTB) antibody (non-specific for both K1IK2 and HSP90).

We further reduced these domains from EIF2AK1 and did co-immunoprecipitation with only the polypeptide NK1 (lacked the kinase insert, kinase 2 and the C-terminal domains) and this peptide was found to associate with purified HSP90 as well (Fig. 5.6). As observed earlier (Fig. 5.4), the deletion of the N-terminal domain did not inhibit EIF2AK1-HSP90 binding. This result thereby highlighted the importance of kinase 1 domain for the formation and maintenance of the EIF2AK1-HSP90 complex.

Further to ensure that kinase 1 domain is the one that binds to HSP90 and not the kinase 2 domain (the catalytic site of EIF2AK1 where eIF2α is phosphorylated), co-immunoprecipitation studies were done with K2. We found that K2 did not bind to purified HSP90 and did not immunoprecipitate with anti-HSP90 antibody (Fig. 5.7).
Fig. 5.6. **NK₁ forms a complex with purified HSP90.** Lane 1 indicates the input level of the two proteins NK₁ and HSP90; Mouse IgG was added as negative control to lane 2; the immunoprecipitate obtained with anti-HSP90 antibody was loaded in lane 3; and lane 4 contained immunoprecipitate obtained with anti-β-actin (ACTB) antibody (non-specific for both NK₁ and HSP90).

This strengthened the assumption that *kinase 1* domain was essential for the association of the EIF2AK1 kinase with HSP90. This assumption stemmed from the fact that HSP90 interacts with several kinases and the possibility of a common binding region could be either of the two kinase domains that are highly conserved among kinases and not the kinase insertion domain, which is a characteristic feature of eIF2α kinases. The *kinase 2* domain, being the catalytic site, is thus proved not to contribute in the binding of EIF2AK1 with HSP90.
**EIF2AK1 and HSP90 interaction**

**Fig. 5.7.** K2 forms a complex with purified HSP90. Lane 1 indicates the input level of the two proteins K2 and HSP90; Mouse IgG was added as negative control to lane 2; the immunoprecipitate obtained with anti-HSP90 antibody was loaded in lane 3; and lane 4 contained immunoprecipitate obtained with anti-β-actin (ACTB) antibody (non-specific for both K2 and HSP90).

### In vitro elf2a kinase activity of EIF2AK1 with HSP90

Purified HSP90 was incubated with recombinant EIF2AK1 and this mix was used for *in vitro* kinase assay to assess the kinase activity of EIF2AK1 in the presence of HSP90 (Fig. 5.8). It was observed that addition of HSP90 to the *in vitro* kinase reaction did not lead to elevation of kinase activity, neither at room temperature nor at the heat-shock temperature of 42 °C which was contrary to what was observed by Pal *et al.*, 1998, where purified native EIF2AK1 from reticulocyte lysate was used. This indicated that the recombinant EIF2AK1 used in the experiment was already in its most active state and saturated for any further trigger like HSP90 which could further enhance its activity towards elf2a phosphorylation.
A. EIF2AK1  -  +  -  +  +  -  +  
HSP90       -  -  +  -  +  +  +  
HS 42       -  -  -  -  +  +  +  

Fig. 5.8. In vitro kinase assay. Effect of purified HSP90 on EIF2AK1 activity. A. Western blot with Phospho eIF2α (eIF2α-P) and eIF2α antibody. Lanes 1-8 consisted of kinase assay mix (with refolded EIF2AK1) with HSP90. B. Densitometric analysis of the blot A. Kinase activity was determined as a measure of eIF2α-P/eIF2α ratio.

B. 

In vivo interaction of EIF2AK1 and HSP90
(Mammalian two-hybrid assay)

In vivo association of EIF2AK1 and HSP90 was done with CheckMate Mammalian two-hybrid (M2H) assay system (Promega). For this, we prepared the constructs pACT-HSP90 and pBIND-HRI by inserting the coding region in the MCS of the empty vectors pACT and pBIND in MluI and KpnI site. The full-length coding sequences of HSP90 and HRI were PCR amplified and cloned in the M2H vectors (Fig. 5.9) such that they formed fusion proteins containing the VP16 and GAL4 protein domains. These constructs were used to transform E. coli Rosetta
BL21(DE3) cells to overexpress and confirm the expression of fusion proteins.

![Restriction digestion of mammalian two-hybrid constructs](image)

**Fig. 5.9. Restriction digestion of the mammalian two-hybrid constructs used for transfection.** The clones were confirmed by restriction analysis and sequencing. The inserts of HRI and HSP90 are indicated in the figure along with their sizes.

The assay constructs were transfected into two cell lines viz. K562 and HeLa, of which K562 is a suspension cell line that belongs to the erythroid lineage with established role of EIF2AK1 while HeLa is an adherent cell line with relatively lesser EIF2AK1 levels as compared to K562 cells. The dual-luciferase assay was done after 24 hrs of transfection and the resultant firefly luciferase readings were normalized to *Renilla* luciferase and plotted as relative luminescence units, RLU (Fig. 5.10).
Fig. 5.10. EIF2AK1 and HSP90 interact with each other in vivo. Dual-luciferase assay was carried out 24 hrs after transfection and the RLU measured for 10 sec at an interval of 1 sec. The Fluc values were normalized with Rluc values and plotted.

As seen in the graph, the experimental values indicate the in vivo interaction of the proteins EIF2AK1 and HSP90. This interaction was more pronounced in the adherent cell line HeLa that could be accounted to higher transfection efficiency and also due to the relative non-abundance of native EIF2AK1 in HeLa cells which could compete with recombinant EIF2AK1 to lessen the reporter transcription in K562 and not so in HeLa cells. This result also supported our in vitro experiments to prove the association of HSP90 with EIF2AK1.

With the association of the two proteins established in vivo, we did further experiments where we transfected K562 and HeLa cells with the constructs and the reporter and after 24 hrs the transfected cells were exposed to lead-treatment for 8 hrs and subjected to heat-shock (42 °C) for 1 hr. The luciferase assay readings were plotted as RLU after normalization with Renilla luciferase assay readings (Fig. 5.11).
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Fig. 5.11. HRI and HSP90 interact with each other under in vivo condition during stress. Dual-luciferase assay was carried out 24 hrs after transfection and the RLU measured for 10 sec at an interval of 1 sec. The Fluc values were normalized with Rluc values and plotted.

As seen in the figure, the luciferase readings for control, lead stress and heat-shock were not significantly different from each other. This marginal difference in luciferase activity suggested that both the stresses did not cause any change in the in vivo association of the EIF2AK1-HSP90 complex. As per our previous studies both these stresses lead to increased eIF2α kinase activity and we had envisaged a change in the association after stress (Matts et al., 1992; Xu et al., 1997; Pal, 1998; Sarkar et al., 2002; Sarkar et al., 2005). The results thus obtained seemed contrary to our previous observations and hence we did further experiments to determine if the maintenance of association was coinciding with modification of the activation status of EIF2AK1. This was done by immunoprecipitation of the EIF2AK1-HSP90 complex using anti-HSP90 MAb and then performing in vitro kinase assay (Fig. 5.12).
A. **K562 cells**

![In vitro kinase assay with Co-IP samples](image)

**Fig. 5.12.** HRI and HSP90 interaction under *in vivo* condition and eIF2α kinase activity during stress. A. *In vitro* kinase assay was carried out with HSP90 immunoprecipitate (that contained co-immunoprecipitated HRI) obtained from K562 and HeLa cell lysate prepared 24 h after transfection. B. Densitometric analysis of the blots in A. Kinase activity was determined as a measure of eIF2α-P/eIF2α ratio.

B.

![Graph](image)

The *in vitro* kinase assay done with the HSP90-EIF2AK1 co-immunoprecipitate confirmed the earlier observations (increase in EIF2AK1 kinase activity) as seen in Fig. 5.12 above. We observed enhanced eIF2α phosphorylation with both lead stress and heat-shock sample. In K562 cells, the eIF2α phosphorylation increased to about 1.5 times during lead stress and heat-shock when compared to control (see Fig. 5.12 B). In HeLa cells, there was 1.5- and 2.5 times increase in eIF2α phosphorylation.
under lead stress and heat-shock, respectively. The variation in the stress response in the two cell lines is attributable to the abundance of native HRI in K562 cells when compared to that in HeLa cells. The results clearly indicate towards modulation of EIF2AK1 kinase activity with simultaneous HSP90 association under the stress conditions mentioned above.

**Discussion**

The co-immunoprecipitation experiments confirmed the binding of full-length EIF2AK1 and its different domains with HSP90 *in vitro*. These results were direct evidence of the association of these two proteins validating the earlier observation from our lab (Pal, 1998) and others (Matts *et al*., 1992; Xu *et al*., 1997). We identified the kinase 1 domain of EIF2AK1 as the domain necessary for the EIF2AK1-HSP90 interaction. The contribution of the kinase 1 domain in the EIF2AK1-HSP90 association is much critical as compared to the other remaining domains of EIF2AK1 that do not associate with HSP90 directly but may have hitherto undefined role in maintenance and modification of this association which cannot be negated by these experiments alone. This binding of one of the two kinase domains of the EIF2AK1 with HSP90 is indicative of the native phenomenon of HSP90 being a chaperone of many of the cellular kinases which would be dependent on conserved regions in these kinases such as the kinase domains. Non-association of the kinase 2 domain also presented
the essentiality of this domain to be available for substrate binding as the kinase 2 domain harbours the ATP binding domain. Though we identified the domain of EIF2AK1 that binds to HSP90, we are still working towards determining the residues that are involved in this association.

The association of EIF2AK1 and HSP90 was also determined in vivo by mammalian two-hybrid assay. The results from these experiments further corroborated the interactions of these two proteins. The reporter luciferase activity increased on the positive association of the two proteins in vivo. Further experiments exhibited that the association did not change when the transfected cells were exposed to stresses viz. lead acetate treatment and heat-shock. This was contrary to what we had expected as the two stresses were established inducers of EIF2AK1 kinase activity. So to affirm whether the stresses caused upregulation of EIF2AK1 activity we co-immunoprecipitated the HSP90-EIF2AK1 complex from these transfected cells and the eluate was used for in vitro kinase assay where the substrate was purified human eIF2α. The in vitro kinase assay confirmed that there was indeed an upregulation of the kinase activity though there was marginal change in the association of HSP90 with EIF2AK1 as determined by luciferase assay. These results when combined suggested that the EIF2AK1-HSP90 complex was somehow modulated during stress to impart kinase activity to EIF2AK1 while maintaining the integrity of the complex. We suggest that the EIF2AK1-HSP90 complex is
present in cells in two states, one that has EIF2AK1 in an activable state and second where EIF2AK1 is already active but still associated with HSP90. We assume the possibility of this second complex to be present in a dynamic conformation state where the active EIF2AK1 could either remain bound in the complex or could be released if the complex becomes conformationally unstable. It is quite intriguing to examine this aspect of the EIF2AK1-HSP90 complex and further study this association in the context of cellular dynamics.