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Regulation of Sam68 Activity by Small Heat Shock Protein 22

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Abstract Sam68 associates with c-*Src* kinase during mitosis. We previously demonstrated that Sam68 functionally replaces and/or synergizes with HIV-1 Rev in rev response element (RRE)-mediated gene expression and virus production. Furthermore, we reported that knockdown of Sam68 inhibited Rev-mediated RNA export and it is absolutely required for HIV-1 production. In the present study, we identified small heat shock protein, *hsp*22, as a novel interacting partner of Sam68. *Hsp*22 binds to Sam68 in vitro and in vivo. Overexpression of *hsp*22 significantly inhibits Sam68-mediated RRE- as well as CTE (constitutive transport element)-dependent reporter gene expression. Furthermore, exposing 293T cells to heat shock inhibits Sam68/RRE function by virtue of elevating *hsp*22. The critical domain of *hsp*22 that interacts with Sam68 resides between amino acids 62 and 133. Our studies provide evidence for the first time that *hsp*22 specifically binds to Sam68 and modulates its activity, thus playing a role in the post-transcriptional regulation of gene expression. J. Cell. Biochem. 99: 1353–1362, 2006.

Key words: Sam68; *Hsp22*; protein–protein interactions; RRE; CTE

Sam68 is an RNA binding protein, which associates with c-Src in mitosis [Fumagalli et al., 1994; Taylor and Shalloway, 1994]. It belongs to a family of proteins that contain KH domains. The KH domain is highly conserved among several RNA binding proteins such as hnRNP K [Siomi et al., 1993a], glycine rich protein 33 [Cruz-Alvarez and Pellicer, 1987], fragile X mental retardation gene product FMR-1 [Siomi et al., 1993b], the *Caenorhabditis elegans* germ-line-specific tumor suppressor GLD-1 [Jones and Schedl, 1995], SLM-1, SLM-2 (Sam68 like mammalian-1 and 2), and the quaking protein QKI-5, QKI-6 and QKI-7 [Ebersole et al., 1996; Chen et al., 1997; Di Fruscio et al., 1999]. Some KH proteins are translational regulators [Saccomanno et al., 1999], while others are deemed to mediate alternative splicing [Stoss et al., 2001; Matter et al., 2002]. Using antisense knockdown strategy, Sam68 has been implicated in cell proliferation and tumorogenesis [Liu et al., 2000].

KH domain is required for RNA-binding, protein–protein interactions and for proper intracellular localization of Sam68 [Chen et al., 1997; McBride et al., 1998]. In addition, Sam68 contains several proline-rich motifs, which are presumed to facilitate the interactions with SH3 domain containing proteins [Chen et al., 1997]. Although Sam68 is localized in the nucleus, it also associates with various signaling molecules in the cytoplasm, including the Src family tyrosine kinases, GRB-2, Fyn and phospholipase Cγ-1 [Richard et al., 1995; Fusaki et al., 1997]. Furthermore, tyrosine-phosphorylated Sam68 was shown to be recruited into signaling complexes of HIV infected peripheral blood mononuclear cells [Najib et al., 2005].

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Sam68 also associates with hnRNP K, involved in RNA metabolism [Yang et al., 2002]. All the above studies suggest that Sam68 is multifunctional protein, with the ability to act as a connecting link between the cytosolic signaling pathways and downstream nuclear pathways, such as RNA synthesis, RNA processing and export.

Although the intrinsic cellular target for Sam68 is unknown, we have previously shown that Sam68 binds to RRE RNA in vitro and in vivo, and can functionally replace and/or synergize with HIV-1 Rev in RRE-mediated gene expression and virus production [Reddy et al., 1999]. These results were subsequently confirmed and extended by others [Derry et al., 2000; Soros et al., 2001; Chen et al., 2001; Li et al., 2002; Yang et al., 2002]. Using an RNAi-based gene knockdown strategy, we have demonstrated that Sam68 is essential for Rev function and is absolutely required for HIV-1 production [Modem et al., 2005]. Sam68 also enhances the activities of Rev-like proteins of other complex retroviruses [Reddy et al., 2000a]. In addition, Sam68 increases Tap-dependent MPMV CTE-mediated gene expression [Reddy et al., 2000b] by promoting the utilization of cytoplasmic RNAs [Coyle et al., 2003]. Additionally, Sam68 has been implicated in the 3′ end processing of unspliced HIV-1 RNAs to be exported to the cytoplasm [McLaren et al., 2004].

To gain insight into the mechanisms of Sam68 action, we sought to identify cellular proteins that are pertinent to its function. Using the C-terminal domain of Sam68 as bait in a yeast two-hybrid screen, we screened a human cDNA library and identified *hsp*22/H11 as a novel interacting partner of Sam68 [Smith et al., 2000; Sun et al., 2004]. *Hsp*22 binds to Sam68 in vitro and in vivo. Overexpression of *hsp*22 inhibits Sam68-mediated RRE- or CTE-dependent transactivation. Thus, our results provide direct evidence that *hsp*22 modulates Sam68 function.

MATERIALS AND METHODS

Plasmids

The construction of Sam68 expression vector was described previously [Reddy et al., 1999]. To generate pGBKT7-Sam68 bait plasmid, human Sam68 cDNA insert (from nucleotide 581 to 1333) was cloned in-frame into the *Bam* HI and *Sal I* sites of yeast DNA binding domain vector (pGBKT7). Wild type and mutant Flag-*hsp*22 (M1 to M5) expression vectors were generated by cloning the PCR amplified products having *Eco* RI and *Bam* HI ends into the cognate sites of Flag-Tag2 vector (Stratagene, San Diego, CA). Details of *hsp*22 primer sequences are available on request. The same cDNA fragments were used to generate the wild type and mutant ACT2-*hsp*22 yeast fusion expression vectors. Cloning of *hsp*22 cDNA bearing *Bam* HI and *Eco* RI ends into the cognate restriction sites of PGX-2T vector yielded the GST-*hsp*22 expression construct.

Yeast Two-Hybrid Screening

pGBKT7-Sam68 was used as bait to screen a pretransformed human brain cDNA library according to the Matchmaker Two-Hybrid System protocol (BD Biosciences Clontech, Mountain View, CA). Isolation, verification, and identification of cDNA clones were done as described previously [Yang et al., 2002].

Cells, Transfections and Chloramphenicol Acetyltransferase (CAT) Assay

293T, HeLa, Cos-1 and U87 were maintained in DMEM supplemented with 10% fetal bovine serum, while Jurkat, SupT1 and U937 were grown in RPMI medium supplemented with 10% fetal bovine serum. In general, between 1 and 3 μg of DNA was transfected into (1×10^5) 293T cells using lipofectamine 2000 (Invitrogen, Carlsbad, CA). The Lac Z vector (0.125–0.25 μg) was used as an internal control. PcDNA3 was used to equalize the amount of DNA in each transfection. At 48-h post-transfection, cells were harvested, and CAT assays were performed as described [Reddy et al., 1999].

RNA Extraction and cDNA Synthesis

Total RNA was extracted from approximately 5×10^6 cells of each type using Trizol® (Life Technologies, San Diego, CA) according to the manufacturer's instructions. cDNA syntheses were performed as previously described [Gerard et al., 1998].

PCR and Quantitative RT-PCR

For the distribution of *hsp*22 mRNA levels in different cell lines, one μg of cDNA was subjected to PCR analysis. Primers used were as follows: *hsp*22 forward, 5′-ATG GCT GAC GGT CAG ATG-3′ and reverse, 5′-TCA GGT

ACA GTG ACT T-3′ to yield a 591-bp product; β-Actin, 5′-GAA ATC GTG CGT GAC ATT AAG-3′ and 5′-CTA GAA GCA TTT GCG GTG GAC GAT GGA GGG GCC-3′ to yield a 450-bp product. PCR products were analyzed on 1% agarose gel and visualized by ethidium bromide staining.

The relative level of *hsp*22 mRNA/cDNA was assessed using the SYBR® Green method as previously described [Gerard et al., 2002]. Primer sequences for *hsp*22 and 18S rRNA were as follows: *hsp*22 forward, 5′-CCA GAG GGT CTG CTG ATC A-3′; reverse, 5′-TCC TGG GGA AGC TCG TT-3′; 18S forward, 5′-CGG CTA CCA CAT CCA AGG AA-3′; and reverse, 5′-GCT CGA ATT ACC GCG GCT-3′. All values were normalized to the 18S rRNA gene and compared to those of untreated cells. Three replicates were performed on each preparation. The mean of three values was determined and the mean result is expressed in the form of mean expression ± standard error (SE).

GST-Pull Down and RNA Electro Mobility Shift Assays

GST or GST-*hsp*22 proteins were purified by standard protocols. Twenty-five milligrams of 293T cell extract was incubated with 25 μl of GST or GST-*hsp*22 bound agarose beads at 4°C for 3 h in 25 μl of binding buffer [Reddy et al., 1999]. The beads were washed four times with 0.25 ml of binding buffer at each time, suspended in SDS buffer, boiled for 5 min, and analyzed by Western blot using rabbit polyclonal antibodies raised against Sam68. The synthesis of ^{32}P UTP-labeled RRE RNA and the RNA-protein binding reactions were carried out as described previously [Reddy et al., 1999].

Co-immunoprecipitation Assay

293T cells (6-well dish) were transfected with Flag-*hsp*22 and Sam68 expression vectors. At 48-h post-transfection, cell extracts were prepared in 1 ml of lysis buffer [Yang et al., 2002]. The lysate was divided into two parts. One portion was incubated with protein-A/G agarose beads and 10 μl of anti-Flag antibodies. The remainder was combined with agarose beads and 10 μl normal mouse IgG. Overnight post-incubation at 4°C, the beads were washed three times with lysis buffer, and immunoprecipitates were suspended in SDS buffer, boiled for 5 min, and analyzed by Western blot using anti-Sam68 or anti-Flag antibodies.

P24 Antigen Capture Assay

For CTE-mediated gag analysis, 293T cells were transfected with pCTE-gag alone, and with Sam68 in the presence or absence of wild type or mutant *hsp*22 expression vectors. At 60-h post-transfection, cell-free supernatants were collected and subjected to p24 antigen assay (Coulter).

RESULTS

Identification of *hsp*22 as a Sam68-Binding Protein by Yeast Two-Hybrid System

Using Sam68 as bait in the yeast two-hybrid system, we screened a human cDNA expression library derived from the human brain and identified several lac-Z activating yeast transformants. DNA from these transformants was isolated and sequenced. A Genbank search revealed that three of the clones are closely homologous to *hsp*22, with alignment beginning at 15 (two clones) and 20 (one clone) amino acids of the 22-kDa protein [Smith et al., 2000; Sun et al., 2004]. The open reading frames of the three clones encode overlapping polypeptides of molecular mass 19 and 20 kDa, all utilizing the yeast activation domain AUG and terminating at a stop codon that precedes a short untranslated sequence. Therefore, these cDNA clones represent N-terminal truncated *hsp*22 genes (two Δ1–15 and one Δ1–20 *hsp*22). The full-length cDNA of *hsp*22 was amplified from 293T RNA and cloned into pACT2 vector.

To investigate whether the interaction between *hsp*22 and Sam68 is specific, yeast cells were co-transformed with pACT2-*hsp*22 alone, or with the corresponding Gal4-DNA binding domain plasmid (pGBKT7) lacking Sam68 cDNA, or with a control vector fused to CEM15 [Sheehy et al., 2002]. Except with Sam68, all failed to activate lac-Z expression, indicating that the interaction between Sam68 and *hsp*22 proteins is specific (Table I). To determine the expression levels of *hsp*22, we performed RT PCR on RNA prepared from seven cell lines: Jurkat, SupT1, U937, 293T, U87, HeLa, and Cos-1. The PCR products were analyzed by 1% agarose gel electrophoresis. All the cell lines were positive for *hsp*22 RNA, suggesting that it is a widely expressed gene (Fig. 1).

TABLE I. Interaction Between Sam68 and *hsp*22 Is Specific

Plasmid combination	Selective medium	β-gal expression
pACT2-*hsp*22	leu(−)	−
pGBKT7-Sam68	Tryp(−)	−
pACT2-*hsp*22 + PGBKT7	Tryp and leu (−)	−
pACT2-*hsp*22 + pGBKT7-Sam68	Tryp and leu(−)	+++
pACT2-*hsp*22 + pGBKT7-CEM15	Tryp and leu(−)	−

0.1 μg of each plasmid DNA indicated in the table was transformed into AH109 yeast and plated on the selective dropout plates. At 4-days post-transformation, transformants were subjected to β-gal filter assay. The transformants graded for β-gal activity are as follows. (+++) dark blue: denotes strong interaction and colonies turned blue within 2 h; and (−) no color: colonies remained white after 2 h.

*Hsp*22 Binds to Sam68 In Vitro and In Vivo

Specific association between Sam68 and *hsp*22 in the yeast-two hybrid assay prompted us to investigate the interaction of these proteins in vitro by GST-pull down assay. For in vitro binding, pGST-*hsp*22 was constructed, and transformation of this vector led to the expression of GST-*hsp*22. 293T cell lysates were used as a source for Sam68 (Fig. 2A, lane 3). To ascertain the levels of GST or GST-*hsp*22 proteins used are comparable, same blot was stained with Coomassie brilliant blue (Fig. 2A, lower panel). As shown in Figure 2A (lanes 1 and 2 of upper panel), Sam68 selectively bound to GST-*hsp*22 but not to GST alone, indicating that these proteins interact in vitro correlating well with the yeast two-hybrid system (Table I).

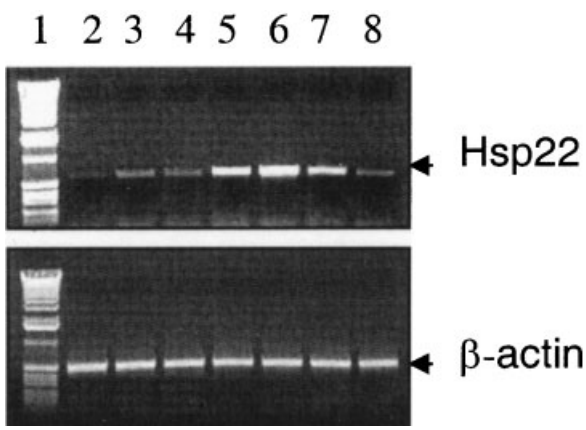

Fig. 1. Distribution of *hsp*22 RNA: Total RNA was isolated from various cell lines and subjected to reverse transcription. The cDNA was subjected to PCR using *hsp*22 and β-actin primers (lane 1, DNA markers; 2, Jurkat; 3, SupT1; 4, U937; 5, 293T; 6, U87; 7, HeLa; and 8, Cos-1). β-actin (**lower panel**) was served to test the integrity of cDNA.

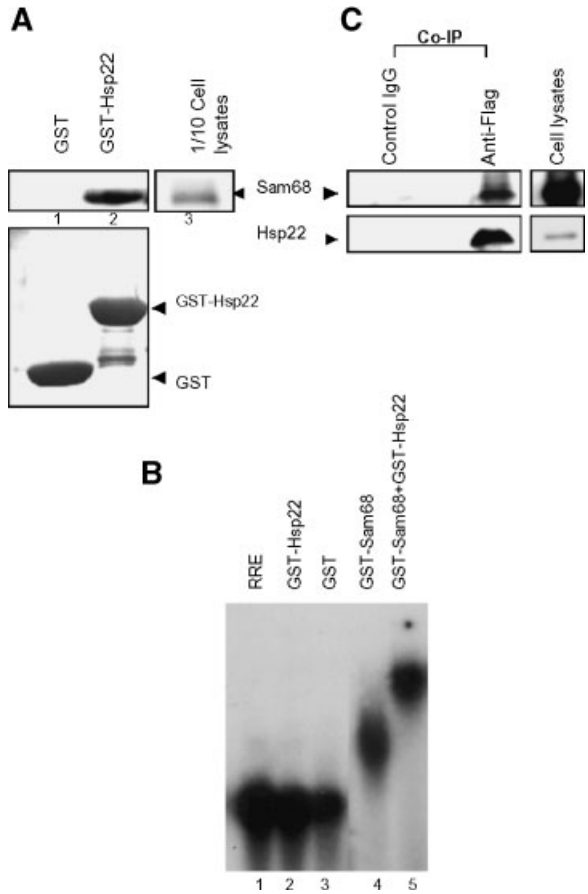

Fig. 2. Sam68 binds to *hsp*22 in vitro and in vivo: **A**: GST-pull down assay: GST and/or GST-*Hsp*22 bound to Sepharose beads were mixed with cell extracts. The bound proteins in parallel with input cell extracts were subjected to immunoblot analysis by anti-Sam68 antibodies. Arrow indicates the Sam68 (**upper panel**). The **lower panel** indicates the levels of GST and GST-*hsp*22, assessed by Coomassie brilliant blue staining. **B**: Electro mobility shift assay, lane 1, ^{32}P labeled RRE RNA alone; lane 2, GST-*hsp*22; lane 3, GST; lane 4, GST-Sam68; and lane 5, GST-Sam68 and GST-*hsp*22. **C**: In vivo association: Anti-Flag antibodies were used to immunoprecipitate Flag-*hsp*22 from 293T cells transfected with pFlag-*hsp*22. Mouse IgG was used as a negative control. Immunoprecipitates were subjected to SDS–PAGE followed by immunoblot analysis using anti-Sam68 antibodies (upper panel). For *hsp*22 detection, same blot was probed with anti-Flag antibodies (lower panel).

We further verified the interaction of *hsp*22 with Sam68 on ^{32}P-labeled RRE RNA in vitro by electro mobility shift assay. Purified GST, GST-Sam68, GST-*hsp*22 and in vitro transcribed ^{32}P-labeled RRE RNA were used in the assay. GST-Sam68 but not GST bound to ^{32}P-labeled RRE-RNA (Fig. 2B, lanes 3 and 4). GST-*hsp*22 by itself did not bind to RRE RNA (Fig. 2B, lane 2). However, when GST-*hsp*22 and GST-Sam68 together were incubated with

labeled RRE RNA, a super shift of the labeled RRE was identified (Fig. 2B, lane 5). These results indicate that *hsp*22 is a directly interacting partner of Sam68 on RRE RNA.

To validate the in vitro binding of Sam68 with *hsp*22 in an in vivo situation in human cells, we performed co-immunoprecipitation assays. *Hsp*22 was expressed as Flag-fusion protein. Anti-Flag antibodies were used to immunoprecipitate Flag-*hsp*22 from 293T cells transfected with pFlag-*hsp*22. Mouse IgG was used as a negative control. The presence of Sam68 in these precipitates was detected by immunoblot analysis, using anti-Sam68 antibodies. Sam68 was detected in the immuno-complexes that were precipitated by anti-Flag antibodies, but not by the control IgG (Fig. 2C), suggesting that *hsp*22 associates with Sam68 in vivo in human cells.

*Hsp*22 Inhibits Sam68-Mediated RRE-Dependent CAT Gene Activity

To explore the functional significance of the in vitro and in vivo associations of *hsp*22 and Sam68, 293T cells were co-transfected with pCMV128 (RRE-CAT) alone, and with pSam68 in the presence or absence of pFlag-*hsp*22. The CAT activity in the cell lysates was determined as described in Materials and Methods. Transfection of pCMV128 alone resulted in very little CAT activity. However, co-transfection of pSam68 increased CAT activity by three-fold over basal levels (Fig. 3A). Co-expression of Flag-*hsp*22 with Sam68 drastically inhibited Sam68/RRE-mediated gene expression, while the *hsp*22 mutant (Fig. 3A) and heat shock factor-1 (data not shown) failed to do so. *Hsp*22 did not inhibit HIV-1 LTR/Tat-driven CAT expression (Fig. 3B). Taken together, these data suggest that *hsp*22-mediated inhibition of CAT gene expression is specific to Sam68 function.

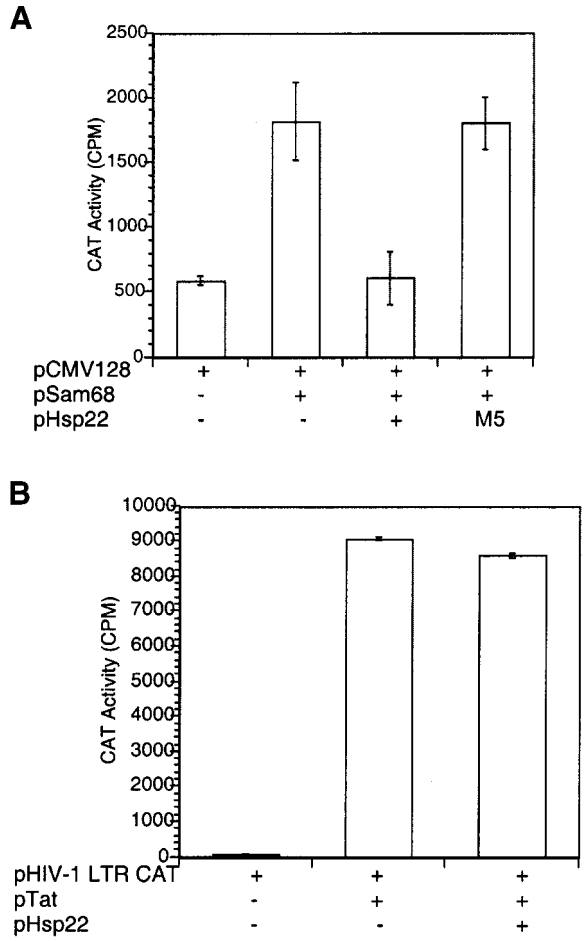

Fig. 3. *Hsp*22 inhibits Sam68 function: 293T cells (2×10^5) were transfected with the expression plasmids as indicated in the figure. **A**: Sam68-dependent RRE-mediated gene expression: pCMV128 (RRE-CAT, 62.5 ng) alone, with Sam68 (62.5 ng) in the presence or absence of wild type or mutant pFlag-*hsp*22 (75 ng); (**B**) HIV-1 LTR/Tat-mediated CAT gene expression: pHIV-1 LTR CAT (125 ng) alone, with Tat (50 ng) in the presence or absence of *hsp*22 (75 ng). pcDNA was used to equalize the amount of DNA in each transfection. Lac-Z reporter plasmid was used as an internal control. CAT assays and separation of reaction products was performed as described in Materials and Methods.

Heat Shock Inhibits the Function of Sam68/RRE

Down modulation of Sam68/RRE function by *hsp*22 prompted us to investigate whether heat shock stress by itself alters the activity of Sam68. For these studies, 293T cells were transfected with pCMV128 in the presence or absence of pSam68 and cells were exposed to 42°C for 2 h prior to harvest at 48-h post-transfection. 293T cells that were not exposed to heat shock served as control. Cell lysates were subjected to CAT analysis as described in Materials and Methods. There was a significant reduction (60%) in Sam68/RRE-mediated CAT activity in the extracts from heat-shocked cells over the control (Fig. 4A). These results led us to hypothesize that thermal stress induces the synthesis of *hsp*22, which in turn modulated the function of Sam68. To test this possibility, the endogenous levels of *hsp*22 RNA from the heat shock subjected cells was measured by real time quantitative RT-PCR from various cells (HeLa, 293T, Cos-1, and U937). *Hsp*22 RNA was increased by several-fold (2.5 to 6-fold) in the cells subjected to heat shock (Fig. 4B). These results suggest that

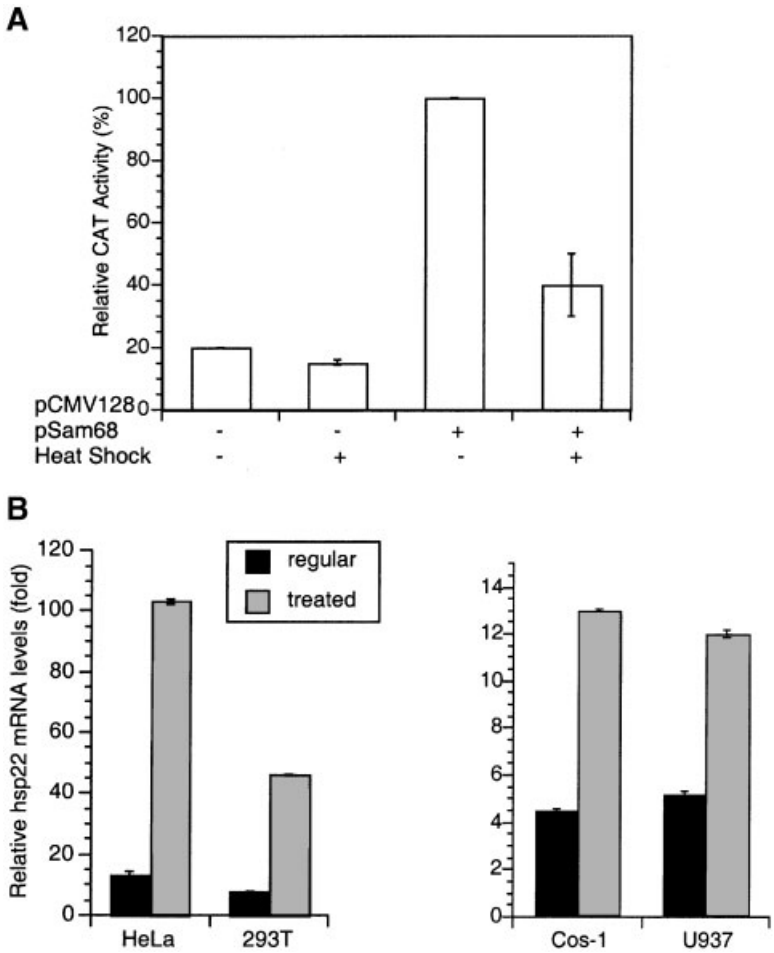

Fig. 4. Effect of heat shock on Sam68 function: (**A**) 293T cells were co-transfected with the expression plasmids as indicated in the figure. Cells were exposed to 42°C for 2 h before harvesting and assayed for CAT activity. In control cells, the Sam68-mediated RRE-dependent CAT gene expression was arbitrarily set to 100%. pCMV128 (62.5 ng) alone, and with Sam68 (62.5 ng). **B**: Heat shock increases *hsp*22 RNA synthesis: 1×10^6 cells were exposed to heat shock at 42°C for 2 h. The total RNA was isolated and subjected to SYBER quantitative real-time PCR analysis as described in Materials and Methods. The RNA from cells that were not exposed to heat shock was used as control.

*hsp*22 induced by heat shock negatively regulates Sam68 activity.

Delineation of Domains of hsp22 Involved in Interaction With Sam68

To map the contact sites for Sam68 on *hsp*22, we constructed yeast activation domain fusion vectors that express several progressive N- and C-terminal deletion mutants of *hsp*22 as shown in the Figure 5A. C-terminal deletion mutants have 360–588 (M1) and 456–588 (M2) bp deleted from their 3′ ends; and the resultant constructs allowed the expression of polypeptides containing 120 and 152 aa of the N-terminal end. N-terminal deletion mutants have 1–186 (M3), 1-282 (M4) and 1–399 (M5) bp deleted from their 5′ ends; the resultant constructs allowed the expression of polypeptides containing 134, 102, and 63 aa of the C-terminal end, respectively. Interactions between *hsp*22 mutants and Sam68 were examined in a yeast two-hybrid assay. Except M5 mutant, all *hsp*22 mutants interacted with Sam68 and activated lac-Z expression (Fig. 5A). Among the *hsp*22 mutants testing positive for Sam68 binding, the interaction with M4 mutant was weak. Taken together, these results suggest that the domain between amino acids 62 and 133 of *hsp*22 is the contact site for Sam68.

To further validate that the effect of *hsp*22 is specific for Sam68, and to correlate the yeast two-hybrid interactions (Fig. 5A) with functional relevance in an in vivo situation, we used CTE-mediated reporter gene expression. 293T

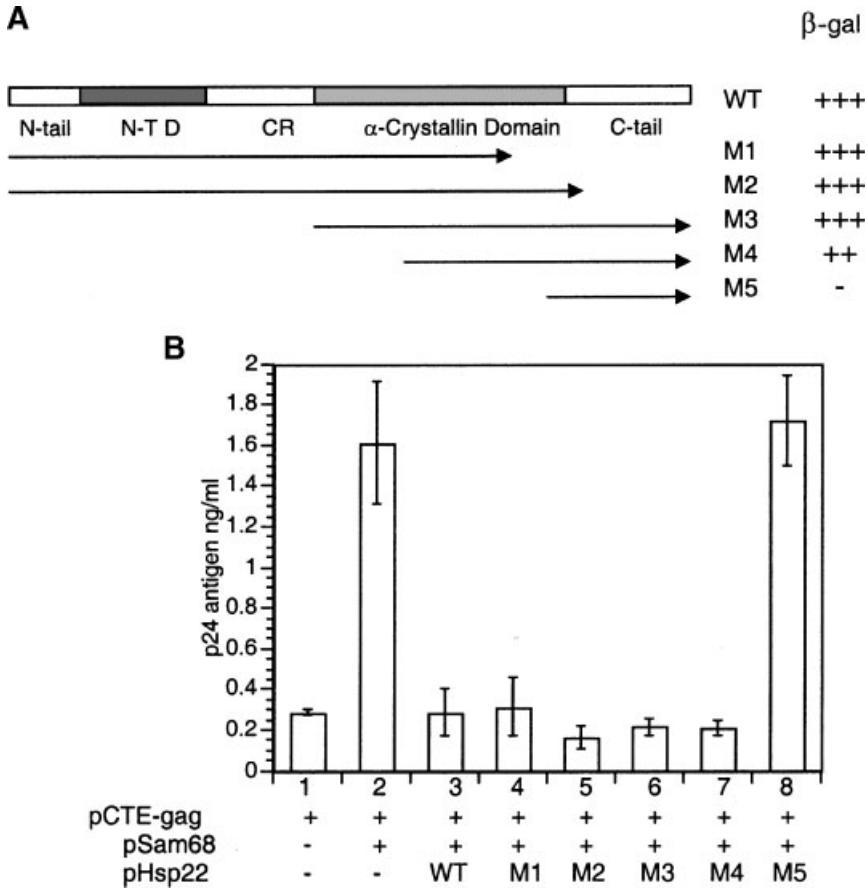

Fig. 5. Mapping the functional domain of *hsp*22 that interacts with Sam68: (**A**) Interaction in the yeast two hybrid assay: Wild type or mutant pACT2-*hsp*22s shown in the figure were co-transformed with pGBKT7-Sam68 into yeast. The transformants graded for β-gal activity is as follows. (+++) Dark blue: denotes strong interaction and colonies turned blue within 2-h; (++) light blue: colonies turned blue after 2 h; and (−) no color: colonies remained white. **B**: Effect of wild type and mutants of *hsp*22 on Sam68/CTE-mediated gag gene expression: 293T cells were transfected with pCTE-gag (250 ng) alone, with Sam68 (500 ng) in the presence or absence of wild type or mutant pFlag-*hsp*22 (100 ng). pcDNA was used to equalize the amount of DNA in each transfection. The Lac-Z reporter plasmid (250 ng) was used as an internal control. At 60-h post-transfection, cell-free supernatants were collected and subjected to p24 antigen capture assay (Coulter).

cells were co-transfected with pCTE-*gag* alone, and with pSam68 in the presence or absence of wild type and mutant Flag-*hsp*22 expression vectors. At 60-h post-transfection, the amount of p24 antigen in cell-free supernatants was measured. pCTE-*gag* alone produced basal levels of p24 antigen (Fig. 5B, lane 1). Co-transfection of pSam68 with pCTE-*gag* increased p24 antigen 5.6-fold (1.61 ng/ml; Fig. 5B, lane 2) over basal levels. However, co-expression of *hsp*22 significantly inhibited the Sam68-dependent CTE-mediated *gag* gene expression (0.285 ng/ml; Fig. 5B, lane 3). With the exception of the M5 mutant (Fig. 5B, lane 8), all other mutants inhibited Sam68/CTE-mediated *gag* gene expression (Fig. 5B, lanes 4 to 7) to the basal levels (0.285 ng/ml). These results indicate that the regulatory domain of *hsp*22, which inhibits Sam68 activity, resides between 62-133 amino acids.

DISCUSSION

Heat shock proteins are a family of intracellular chaperone molecules that facilitate protein folding and assembly [review by Santoro, 2000]. These stress proteins are selectively synthesized following various stimuli, including heat and viral infections [review by Brenner and Wainberg, 2001]. Recent studies reveal that Hsp70 plays a significant role at various stages of HIV-1 life cycle, that is, nuclear import and transcription [review by Brenner and Wainberg, 2001]. Conversely, the role of small heat shock proteins, such as *hsp*22, either in the retroviral life cycle or in cellular processes is unexplored.

We report here for the first time that Sam68 interacts with *hsp*22. *Hsp*22 was originally identified as a protein kinase homologous to the large subunit of Herpex simplex virus type-2 ribonucleotide reductase (ICP10) [Smith et al., 2000]. Subsequently, based on phylogenetic analysis and its ability to heterodimerize with *hsp*27, *hsp*22 was categorized as a member of small Hsp family [Sun et al., 2004]. Thus, the biological role of *hsp*22 is controversial. Nevertheless, identification of several cDNA clones that encode *hsp*22, and its association with Sam68 confirmed in three independent biochemical assays (Fig. 2), unambiguously substantiates that the interactions between Sam68 and *hsp*22 are specific. Also, our current study, which employed 293T cells to obtain full-length cDNA of *hsp*22, unequivocally confirms that *hsp*22 is indeed expressed in 293T cells. These results are in direct contrast to the report by Smith et al. [2000], in which they have reported the absence of *hsp*22 in 293T, HeLa and Cos-1 cells. However, Chowdary et al. [2004] have recently reported that HeLa cells also express *hsp*22. The reason for the disparity between the report by Smith et al. [2000] and these other studies is unclear. However, it is possible that cells from different sources might have contributed for this discrepancy.

Our studies provide evidence that overexpression of *hsp*22 inhibited the activity of Sam68 in two independent reporter gene expression assays, that is, RRE and CTE (Figs. 3 and 5). The inhibitory effect of *hsp*22 on Sam68-mediated RRE- and CTE-directed gene expression is likely due to direct protein–protein interactions, as *hsp*22 itself failed to bind to RRE RNA (Fig. 2B). Previously, it was shown that hnRNP K binds to Sam68 and inhibits its function by a similar mechanism [Yang et al., 2002]. However, it is important to note that the Sam68 domains interacting with *hsp*22 and hnRNP K partially overlap. Both the KH and C-terminal tyrosine-rich domains of Sam68 are the contact sites for hnRNP K [Yang et al., 2002]; the latter domain is dispensable for interaction with *hsp*22, since it interacts equally and efficiently with the transdominant negative Sam68 mutant [Reddy, 2000; data not shown]. Since *hsp*22 does not inhibit Sam68 binding to RRE RNA, it is conceivable that binding of *hsp*22 to the KH domain of Sam68 blocked the function of Sam68. It is also important to note that the inhibition of Sam68 activity by *hsp*22 was not due to the down modulation of Sam68 expression (data not shown). *Hsp*22 also inhibited the function of Rev and its synergistic effect with Sam68 (data not shown). We reason that not only Sam68 knockdown renders cells defective for Rev-mediated RNA export [Modem et al., 2005], but also the reduction of endogenous Sam68 activity by *hsp*22 may eventually impact Rev function as well.

Similar to other small Hsps, *hsp*22 comprises of several structural domains: a highly conserved α-crystallin domain, a less conserved N-terminal domain (N-T D), a variable central region (CR), and variable N- and C-terminal tails [Fig. 5A, Sun et al., 2004]. *Hsp*22 forms homo-dimers through N–N and N–C interactions, while it forms hetero-dimers through the α-crystallin portion of its C′ domain [Sun et al., 2004]. Our mapping studies indicated that the N-terminal (1 to 62) and C-terminal (133–196) amino acids of *hsp*22 are dispensable for Sam68 binding, and this observation correlates well with the inhibitory function (Fig. 5B). Interestingly, this domain consists of a consensus Src homology motif (PPPFPGEP; amino acids 88–95) within the α-crystallin domain implicated in protein–protein interactions [Smith et al., 2000; Sun et al., 2004]. Since the KH and proline-rich domains of Sam68 mediate protein–protein interactions, it is possible that a complex formed between the Src-homology/α-crystallin domains of *hsp*22, and the KH/proline motifs of Sam68 blocked the activity of Sam68.

*Hsp*22 is a cytoplasmic protein [Yu et al., 2001], while Sam68 is a nuclear protein. However, Sam68 does not seem to shuttle in human cells [Soros et al., 2001]. This raises a question of how a cytoplasmic protein modulates the function of nuclear protein. It is possible that overexpressed *hsp*22 blocks the nuclear import of de novo-synthesized Sam68, thus resulting in inhibition of Sam68 activity. In light of these findings, future studies should be directed towards understanding the role of small heat shock proteins in the post-transcriptional regulation of HIV-1 gene expression.

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