

Functional interaction of Sam68 and heterogeneous nuclear ribonucleoprotein K

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Sam68 is a target of the c-Src tyrosine kinase. We previously showed that overexpression of Sam68 functionally substitutes for, as well as synergies with, HIV-1 Rev in Rev-response element (RRE)-mediated gene expression and virus replication. Here we describe the identification of heterogeneous nuclear ribonucleoprotein K (hnRNP K) as a protein that specifically interacts with Sam68 *in vitro* and *in vivo*. hnRNP K did not bind to RRE-RNA directly, but formed a super complex with Sam68 and RRE *in vitro*. RNase treatment did not change the strength of binding of hnRNP K to Sam68. We demonstrated that hnRNP K significantly inhibited Sam68-mediated, but not Rev-mediated, RRE-dependent gene expression. We further showed that Sam68, but not a non-functional mutant Sam68p21, inhibited transcriptional activation of CT element by hnRNP K. Interestingly, the Sam68p21 with a single amino acid substitution in the nuclear localization domain exhibited less affinity for hnRNP K *in vitro*. We propose that the direct interaction of Sam68 and hnRNP K adversely affect the activities of both proteins in signal transduction pathways of both transcriptional and post-transcriptional events.

Oncogene (2002) 21, 7187–7194. doi:10.1038/sj.onc.1205759

Keywords: Sam68; hnRNP K; RRE; CT element; protein–protein interaction

Introduction

Sam68 is a nuclear RNA-binding protein and a cell cycle regulated phosphorylation target of c-Src and Cdc2 kinases (Taylor *et al.*, 1995; Resnik *et al.*, 1997; Fumagalli *et al.*, 1994). The 443 amino acid protein is comprised of two putative RNA binding domains, namely, an RGG box and a KH (for hnRNP K

homology) domain. The KH domain is highly conserved among several RNA binding proteins, such as hnRNP K (Siomi *et al.*, 1993), fragile X mental retardation gene product (FMR-1) (Siomi *et al.*, 1994), and the *Ceanorhabditis elegans* germline-specific tumor suppressor GLD-1 (Jones and Schedl, 1995). It is essential for self-association, RNA binding as well as cellular localization of wild type Sam68 (Chen *et al.*, 1997; McBride *et al.*, 1998). *In vitro* selection of RNA ligands has shown that Sam68 can bind to certain RNA sequences with high affinity and specificity (Lin *et al.*, 1997). Sam68 contains five proline-rich motifs, which have been shown to facilitate binding to SH3 domain containing proteins. A nuclear localization signal (NLS) has been mapped to the C-terminal tyrosine-rich region of Sam68 (Ishidate *et al.*, 1997). Sam68 associates with a variety of signaling molecules, including members of the Src family tyrosine kinases, growth factor receptor-bound protein 2 (GRB-2) and phospholipase C γ -1 (Fusaki *et al.*, 1997; Richard *et al.*, 1995). These data suggest that Sam68 functions as a multifunctional SH3 and SH2 adapter protein, with the ability to link cytosolic signaling pathways to downstream effects involved in RNA metabolism, such as transcription, RNA processing and RNA transport.

We have recently implicated Sam68 in the post-transcriptional regulation of all complex retroviruses (Reddy *et al.*, 1999, 2000a). We demonstrated that Sam68 binds to the Rev-response element (RRE) of human immunodeficiency virus 1 (HIV-1) *in vitro* and *in vivo*, and functionally replaces as well as synergies with HIV-1 Rev in RRE-mediated gene expression and virus replication (Reddy *et al.*, 1999). Sam68 also enhances the effects of HTLV-1 Rex protein as well as the Rev-like proteins of other lentiviruses (Reddy *et al.*, 2000a). Interestingly, mutations in the C-terminus of Sam68 led to a dominant negative phenotype in these activities (Reddy *et al.*, 1999).

To gain more insight into the mechanisms of Sam68 action, we searched for cellular proteins that might functionally interact with Sam68. Using the C-terminus of Sam68 as bait in a yeast two-hybrid screen, we identified several novel protein partners, including hnRNP K and some SH3-containing proteins such as the scaffold attachment factor B (SAF-B) (Argraves *et*

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Received 20 November 2001; revised 4 June 2002; accepted 14 June 2002

al., 1990) and fibulin (Renz and Fackelmayer, 1996). In this study, we describe the functional interaction between Sam68 and hnRNP K in the transcriptional and post-transcriptional regulation of gene expression.

Results

Sam68 interacts with hnRNP K in vitro and in vivo

We screened a human placenta cDNA library by the yeast two-hybrid system to identify proteins that interact with the Sam68 C-terminus, which includes the three proline-rich domains and a nuclear localization domain. Approximately 3.5×10^5 HF7C yeast transformants were plated in the absence of tryptophan, leucine, histidine and in the presence of 25 mM 3-aminotriazole and were screened for the expression of *Gall-lacZ* by β -galactosidase assay. Each plasmid rescued from β -galactosidase positive clone was co-transfected with the bait plasmid into yeast and confirmed the specific interaction by β -galactosidase assay. A total of 25 positive clones were thus identified and further characterized by DNA sequencing.

Most of the positive isolates represented repeated isolates of SH3 domain-containing cDNA sequences. Among them, two independent clones were derived from the C-terminus of hnRNP K cDNA (amino acids 160–463) encompassing a KH domain, a proline-rich SH3-binding domain (SBD) and the hnRNP K nuclear shuttling domain (KNS) (Michael *et al.*, 1997). We were interested in the interaction of Sam68 and hnRNP K as both proteins are RNA-binding proteins that are involved in RNA metabolism.

To confirm the interaction between Sam68 and hnRNP K, we tested the *in vitro* binding of recombinant Sam68 and hnRNP K proteins to each other. GST and GST-Sam68 fusion proteins were expressed in *E. coli*, purified by glutathion-sepharose beads, and incubated with *in vitro* translated ^{35}S -labeled luciferase (negative control) or ^{35}S -labeled hnRNP-K. After incubation, the beads were washed extensively and the bound materials were analysed by SDS-PAGE followed by autoradiography. As shown in Figure 1a, hnRNP K strongly interacted with Sam68 (lane 6). No interaction was observed between Sam68 and luciferase proteins, or between hnRNP K and GST proteins (lanes 3 and 5 respectively).

The interaction between Sam68 and hnRNP K was also detected when GST-hnRNP K was bound to beads and HeLa cell extracts were used as a source of Sam68 in the binding experiments (Figure 1b, lane 3). Since both Sam68 and hnRNP K are RNA-binding proteins, we next tested whether the interaction between the two proteins was mediated by RNA. As shown in Figure 1b, lane 5, RNase treatment did not change in the strength of binding hnRNP K to Sam68, indicating that the interaction between Sam68 and hnRNP K was direct.

We also used a gel mobility assay to assess the interaction Sam68 and hnRNP K. Purified GST, GST-Sam68 and GST-K proteins were used in the gel shift

assay. ^{32}P -labeled RRE-RNA bound to GST-Sam68 but not to GST, as reported previously (Figure 1c, lanes 4 and 2). GST-K itself did not cause the RRE-probe to shift significantly. However, when GST-Sam68 and GST-K proteins were first incubated together before adding the labeled RRE-RNA, a supershift of the RNA was observed. These findings suggest that hnRNP K complexes with Sam68 and RRE-RNA.

Further, we carried out a co-immunoprecipitation assay to assess whether hnRNP K interacts with Sam68 *in vivo*. 293 T cells were co-transfected with Flag-tagged hnRNP K or Flag expression vectors in the presence of pSam68. The total cellular proteins from these cells were immunoprecipitated with Flag antibodies and the precipitates were subjected to Western blot analysis using antibodies to Sam68. Sam68 was only detected with antibodies to Flag in the complexes from the lysates made from the cells expressing Flag-hnRNP K, but not with control Flag vector (Figure 1d, upper panel) indicating that hnRNP K interacts with Sam68 *in vivo*. The same blot was stripped and performed Western blot analysis using anti-Flag antibodies to show the expression of hnRNP K (lower panel).

Redistribution of Sam68 and hnRNP K after actinomycin D treatment

To investigate the functional connection of Sam68 and hnRNP K *in vivo*, we examined whether Sam68 and hnRNP K have similar dynamic movements in response to treatment with actinomycin D, a transcription inhibitor (Reich *et al.*, 1962). HeLa cells were co-transfected with GFP-Sam68 or GFP-Rev and FLAG-tagged hnRNP K expression constructs. Twenty-four hours post-transfection, cells were treated with or without 5 $\mu\text{g/ml}$ actinomycin D, a concentration known to inhibit RNA polymerases I and II, for 3 h. Then the cells were fixed and stained with anti-FLAG antibodies and examined under a confocal microscope. GFP-Sam68 was localized in the nucleus with greater concentration in two speckles, in agreement with previous observations (Chen *et al.*, 1999). hnRNP K was localized evenly in the nucleoplasm, excluded from the nucleoli. Upon treatment with actinomycin D, the concentrations of GFP-Sam68 became more fragmented in the nucleoplasm while hnRNP K also became more condensed throughout the nucleus. As a control, GFP-Rev was found to relocalize from the nucleus to the cytoplasm in response to treatment with actinomycin D, as expected. These findings showed that Sam68 and hnRNP K proteins have a similar dynamic movement in response to actinomycin D, and support the physiological interaction of the two proteins *in vivo*.

hnRNP K inhibits Sam68-mediated RRE-dependent gene expression

We next investigated the possible cross-talk between Sam68 and hnRNP K in RRE-mediated gene expres-

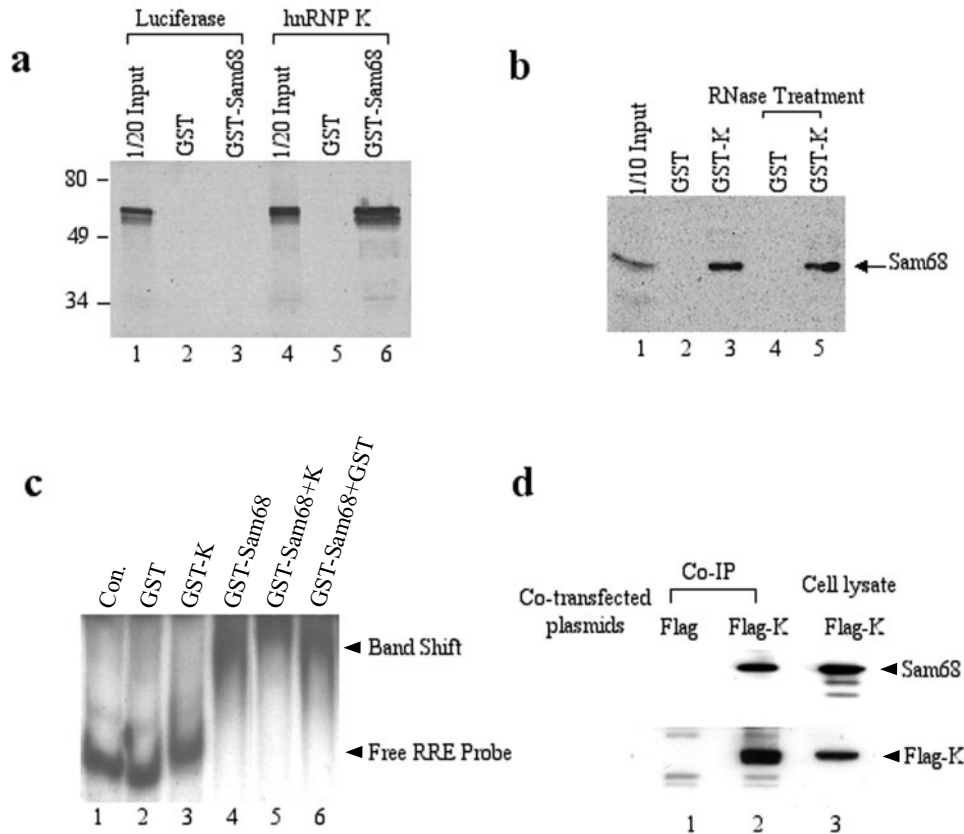


Figure 1 Interaction between Sam68 and hnRNP K *in vitro* and *in vivo*. (a) ³⁵S-methionine-labeled hnRNP K and luciferase translated *in vitro* were incubated with matrix-bound GST or GST-Sam68 for the binding reactions. After washing the beads, the eluted proteins were analysed by SDS-PAGE and autoradiography. One out of 20 of the labeled hnRNP K and luciferase translated *in vitro* was also analysed. (b) HeLa cell extract was either treated or untreated with an RNase mixture (Ambion) consisting of 500 U/ml RNase A and 20 000 U/ml RNase T1 for 30 min before being used in the binding assay. The bound proteins were run in parallel with a sample of one out of 10 of the input cell extract for Western blotting and immuno-detected with anti-Sam68 antibody. The Sam68 band is indicated by an arrow. (c) GST, GST-Sam68 and GST-K fusion proteins were purified by incubation with glutathion-Sepharose beads and eluted with 20 mM glutathion. Typically, 1×10^4 c.p.m. of ³²P-labeled RRE-RNA and 100 ng of each protein was used. For lanes 5 and 6, GST-Sam68 was pre-incubated with GST or GST-K in the binding buffer for 15 min before the probe was added. The reaction mixture was separated by 4.5% non-denaturing PAGE and exposed directly to X-ray film. (d) *In vivo* binding of hnRNP K to Sam68. Flag antibodies were used to immunoprecipitate interacting components from the lysates prepared from the 293T cells transfected with Flag-tagged hnRNP K and Sam68 (3 μ g each), Flag vector and Sam68 (3 μ g each) expression vectors. The immunoprecipitates were subjected to SDS-PAGE followed by Western blot analysis using Sam68 antibodies (upper panel, lanes 1: Flag, lane 2: hnRNP K; and lane 3: lysate prior to immunoprecipitation and arrow indicates Sam68). To assess the expression of Flag-hnRNP K, the same blot was probed with anti-Flag antibodies (lower panel, arrow indicates Flag-K)

sion *in vivo*. Previously we have shown that Sam68 activates RRE-dependent reporter gene expression (Reddy *et al.*, 1999). We transfected 293T cells with Sam68, Rev or hnRNP K expression plasmids together with an RRE-dependent reporter plasmid pCMV128. Forty-eight hours post-transfection, cells were harvested and cell extracts were used for CAT assays. As shown in Figure 3a, Sam68 and Rev enhanced RRE-mediated gene expression from the basal level 21- and 56-fold respectively, consistent with our previous reports (Reddy *et al.*, 1999). HnRNP K alone modestly inhibited the CAT activity. Co-expression of Sam68 and hnRNP K resulted in dose-dependent inhibition of Sam68-mediated gene activation. In contrast, Rev-mediated gene expression was slightly enhanced by hnRNP K. The effect of hnRNP K on RRE reporter system was specifically since no effect was seen on the

internal control reporter (data not shown). Using another reporter system, pSV-gagpol-MPMVCTE (Bray *et al.*, 1994), we also observed a strong inhibition of Sam68-mediated CTE-dependent gag expression by hnRNP K (Figure 3b). These data suggested that hnRNP K specifically inhibited Sam68 but not Rev activation.

Sam68 inhibits hnRNP K transcriptional activation

HnRNP K has been reported to activate transcription from the sequence CCCTCCCCA, known as the CT element of the human *c-myc* promoter gene (Michelotti *et al.*, 1996; Tomonaga and Levens, 1995). To determine whether Sam68 and hnRNP K might interact at multiple regulatory levels, we tested the effect of co-expressing hnRNP K and Sam68 on CT-

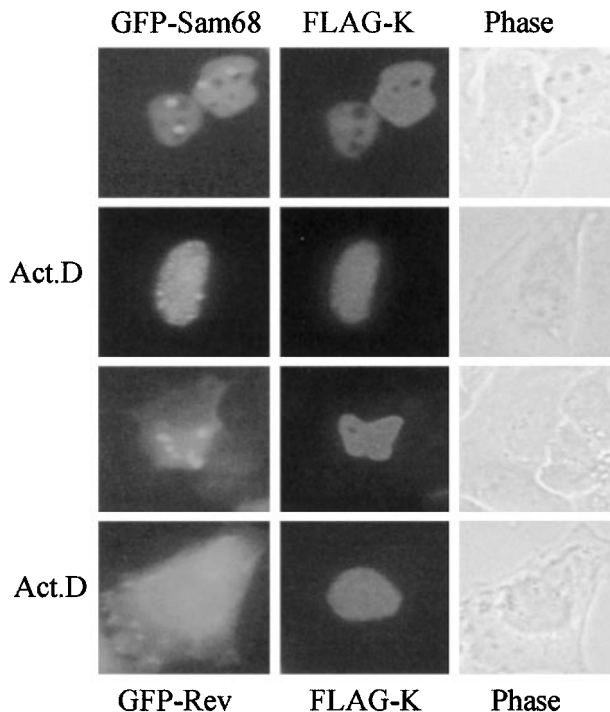
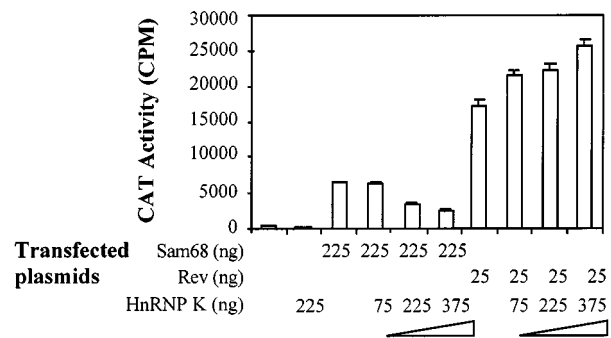


Figure 2 Sam68 and hnRNP K are nuclear proteins with transcription-independent nuclear localization. HeLa cells were co-transfected with expression vectors encoding FLAG-tagged K with GFP-Sam68 or GFP-Rev. At 24 h post-transfection, the medium was replaced with either fresh medium (control) or medium containing 5 μ g/ml actinomycin D (Act. D) and incubation was continued for another 3 h prior to fixation and immunostaining with anti-FLAG antibody

directed reported gene expression. In these experiments, we used plasmids expressing an N-terminal deleted mutant of Sam68 (Sam68 Δ 96), which is fully functional for activation of RRE (Reddy *et al.*, 1999), and Sam68p21, a dominant negative mutant that inhibits RRE-mediated gene expression (Reddy, 2000b). These plasmids were co-transfected with a plasmid encoding the *cat* reporter gene driven by a synthetic promoter containing three CT element repeats (Tomonaga and Levens, 1995) into 293T cells. Forty-eight hours post-transfection, the cells were harvested and the cell extracts were used to detect the CAT activity. As shown in Figure 4a, consistent with previous reports, expression of hnRNP K only slightly activated transcription from the reporter construct containing the CT3 promoter element, presumably due to the high level of endogenous hnRNP K protein (Shnyreva *et al.*, 2000). However, co-expression with the functional Sam68 Δ 96 dramatically inhibited CAT gene expression in the presence or absence of exogenous hnRNP K. In contrast, the Sam68p21 mutant increased gene expression in the presence or absence of hnRNP K. These effects are considered sequence specifically as no effect was seen on the internal control reporter (Figure 4b). Western blots assays using the same cell extracts used in CAT assays (Figure 4a) probed with anti-Sam68 antibodies

a



b

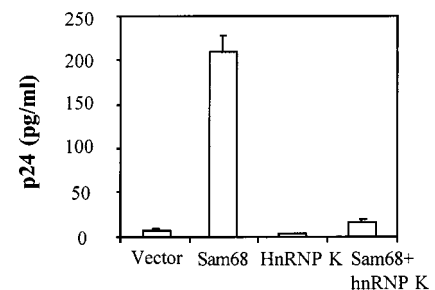


Figure 3 hnRNP K inhibits Sam68-mediated RRE-dependent reporter gene expression. **(a)** 293T cells were co-transfected with the indicated combinations of each pCMV128 (100 ng), Rev (25 ng), Sam68 (225 ng) and hnRNP K (75–375 ng) expression plasmids. pCDNA-LacZ expression vector was co-transfected as an internal control of transfection efficiency. Forty-eight hours post-transfection, cell extracts were prepared and subjected to CAT assay as described in Materials and methods. **(b)** 293T cells were transfected with gagpal-CTE (200 ng) and Sam68 (600 ng) or with hnRNP K (600 ng) expression plasmids. The expression level of p24^{gag} was measured 48 h post-transfection

and anti-FLAG antibodies respectively confirmed that both Sam68 and hnRNP K proteins were expressed at comparable levels (Figure 4c). These results demonstrated that Sam68 inhibited hnRNP K-mediated transactivation of the CT-promoter.

Sam68p21 showed decreased binding to hnRNP K

It was of interest to note that the single point mutation in Sam68p21 abolished the inhibition of the CT3-reporter expression as well as transactivation of Rev/RRE-mediated expression (Figure 4a; Reddy, 2000b). To explore the underlying mechanism, we examined the ability of GST-Sam68 GST-Sam68p21 fusion proteins to bind hnRNP K. Extracts from 293T cells transfected with FLAG-tagged hnRNP K were used as a source of hnRNP K protein. As shown in Figure 5, upper panel, GST-Sam68 bound strongly with hnRNP K while the control GST did not, further supporting the experiments shown in Figure 1. Interestingly, the mutant Sam68p21 had a much weaker binding to hnRNP K protein comparing the wild-type Sam68. To confirm that equal amount of GST fusion proteins were used,

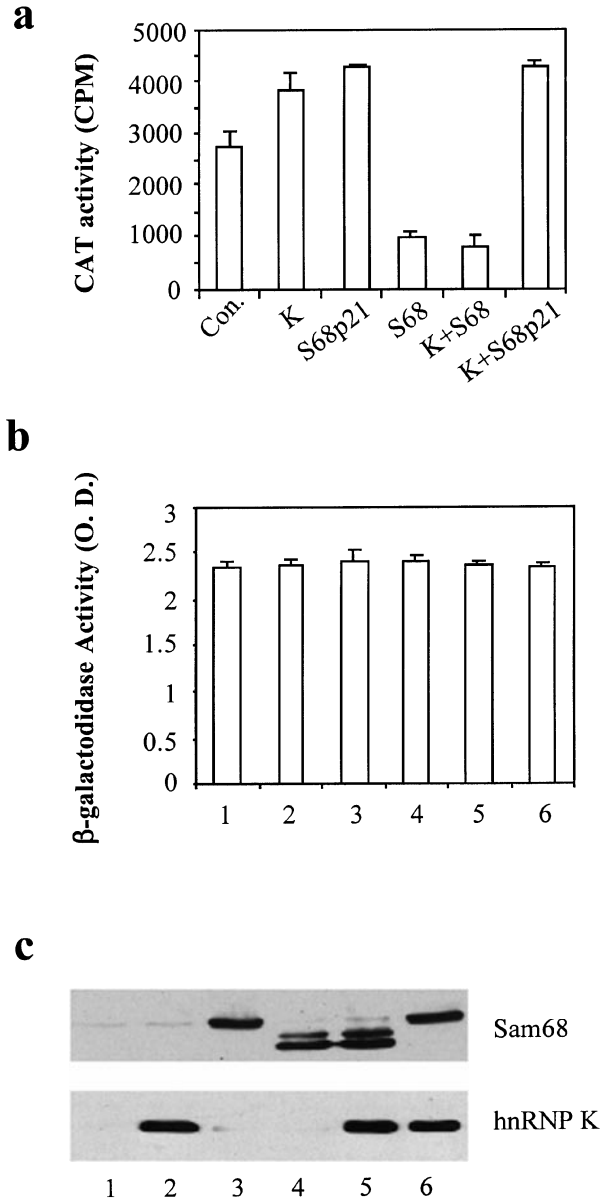


Figure 4 Sam68 inhibited the transactivation of hnRNP K on a CT element-containing promoter. (a) 293T cells were co-transfected Δ 56-CT3 reporter construct (500 ng) with the indicated combination of FLAG-tagged hnRNP K (500 ng), Sam68 (500 ng), Sam68p21 (500 ng) expression plasmids. PcDNA-LacZ expression vector was co-transfected as an internal control of transfection efficiency. Forty-eight hours post-transfection, cell extracts were prepared and subjected to CAT assay as described. (b) The same cell extracts in (a) were tested for β -galactosidase activity. The average values of optical density (O.D.) were indicated. (c) The same cell extracts as in (a) were run on SDS-PAGE and subjected to Western blotting using anti-FLAG and anti-Sam68 antibodies

the membrane was stripped and re-probed with Sam68 antibodies. As shown in the lower panel, indeed, the same amount of GST-Sam68 and GST-Sam68p21 were used and both bind equally to the endogenous Sam68. These data strongly suggest that the nuclear localization domain of Sam68 is involved in the interaction of

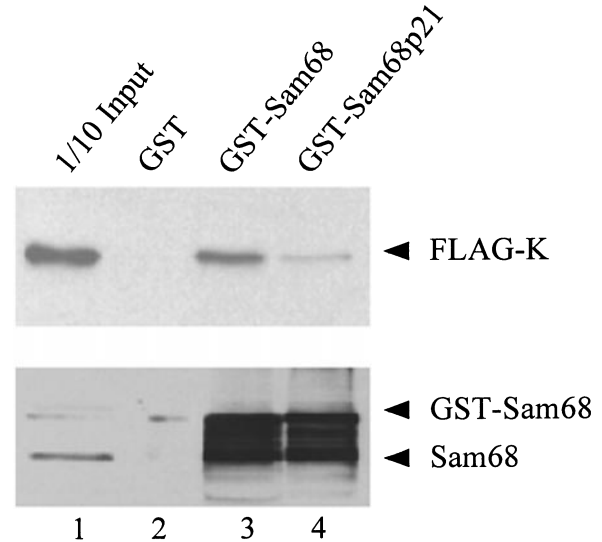


Figure 5 A single point mutation in the nuclear localization domain of Sam68 decreased the affiliation to hnRNP K. Cell extract from 293T cells transfected with FLAG-tagged hnRNP K expression plasmids was used as the source of hnRNP K in a binding assay. The bound proteins were immunodetected by anti-FLAG antibody (upper panel). The FLAG-K band is indicated by an arrow. The membrane was stripped and re-probed with anti-Sam68 antibody (lower panel). GST-Sam68 and endogenous Sam68 were indicated by arrows

Sam68 and hnRNP K, and that this interaction may be important in the observed effect of Sam68 on CT3-reporter gene expression.

Discussion

We describe here the functional interaction between Sam68 and hnRNP K proteins. HnRNP K was first shown to be one of the most abundant components of the heterogeneous nuclear ribonucleoprotein (hnRNP) particle but its function has not been clearly defined (Matunis *et al.*, 1992). HnRNP K is known to interact with many proteins involved in several cellular processes (e.g. signal transduction and gene expression), possibly serving as a ‘docking platform’ mediating cross-talk between these molecules (Bomsztyk *et al.*, 1997).

Both Sam68 and hnRNP K are RNA-binding proteins containing a conserved KH domain responsible for protein-protein interaction. Interestingly, the KH domain of Sam68 was not solely, if at all, involved in binding to hnRNP K, since a single point mutation in the C-terminal nuclear localization domain of Sam68 significantly impaired this binding (Figure 5). Furthermore, the Sam68 bait we used in the yeast screening was the C-terminus of Sam68 without KH domain. The interaction between Sam68 and hnRNP K is direct, as RNase treatment had no effect on the binding (Figure 1b). We have also demonstrated that these proteins interact with each other *in vitro* by pull-down assay and *in vivo* by immunoprecipitation assay (Figure 1b,d). The common nucleoplasmic localization

of Sam68 and hnRNP K, and the similar effect of transcription inhibitors on this localization suggest that both proteins are involved in RNA metabolism, including transcription, processing and transport of newly synthesized RNAs.

Our data provided evidence that Sam68 and hnRNP K functionally interact *in vivo*. The inhibitory effect of hnRNP K on Sam68-mediated RRE-directly gene expression is likely due to direct protein–protein interaction as hnRNP K itself did not bind to RRE–RNA (Figure 1c). Since Sam68 still binds RRE–RNA in the presence of hnRNP K (Figure 1c), it is possible that binding of hnRNP K to the C-terminal of Sam68 blocked the function of Sam68. It is intriguing to find that hnRNP K enhanced the Rev-mediated RRE-dependent gene expression. The reason for the opposite effects on RRE-dependent gene expression mediated by Rev and Sam68 is not known. It has been suggested that the nuclear export pathway mediated by Sam68 and Rev are distinct as evidenced by their differential sensitivity to leptomycin B, an inhibitor of CRM-1 (Reddy *et al.*, 1999). Recently, we also found that several additional KH-containing proteins could enhance Rev-mediated RRE-dependent gene expression (data not shown). hnRNP K was reported to increase RNA synthesis from the trans-activation reporter gene (Lee *et al.*, 1996). Taken together, our findings suggest that Sam68 may influence RNA metabolism not only by means of its RNA binding domain but also through its interaction with other nuclear proteins.

On the other hand, Sam68 down-modulates the stimulatory effects of hnRNP K on gene expression. It is noted that expression of hnRNP K can both activate and repress RNA polymerase II promoters (Michelotti *et al.*, 1996; Tomonaga and Levens, 1995; Maiu *et al.*, 1998). Previous studies showed that transcriptional activation by hnRNP K is dependent on the structure of the promoter and is not limited to the CT element found in the *c-myc* promoter (Lee *et al.*, 1996). The finding that hnRNP K co-immunoprecipitates with epitope-tagged TFIID TATA-binding protein from nuclear extracts has provided the evidence for the activation of hnRNP K (Michelotti *et al.*, 1996). Our results demonstrated that Sam68 could antagonize this activity of hnRNP K. It is interesting to find out that the Sam68 mutant with a single point substitution in the nuclear localization domain exhibited less affinity for hnRNP K *in vitro* and did not inhibit the activation by hnRNP K. However, this mutant Sam68, Sam68p21, did not lose affinity to Sam68 comparing to wild-type Sam68 (Figure 5) as well as its binding to RRE–RNA (data not shown). It is supported by the fact that the KH domain of Sam68 is responsible for its self-association and RNA binding. Further, Sam68p21 was previously shown to localize to the cytoplasm of transfected cells and exerted a dominant negative effect on RRE-mediated gene expression and HIV replication (Reddy, 2000b). Our data strongly suggest that the inhibitory effect of Sam68 on hnRNP K action on CT element is through direct protein–protein interaction.

We demonstrated that the interaction of Sam68 and hnRNP K is antagonistic to their respective activities. Since Sam68 and hnRNP K bind to different RNA or DNA sequences, it is possible that Sam68 and hnRNP K could assemble multiple factors on RNA or DNA and allow multilateral cross-talk among these components. Our observations provide an exciting avenue for investigating this multilateral cross-talk involved in intracellular signal transduction pathways.

Materials and methods

Construction of plasmids

Plasmids were constructed by standard methods. Sam68 fragment (amino acids 292–443) was cloned into pAS2-1 (Clontech) to generate plasmid pAS2-1 Sam68 (292–443) and was used for the yeast two-hybrid screening. For generation of FLAG-tagged hnRNP K, the DNA fragments were amplified by PCR and cloned in-frame into pCMV-FLAG vectors (Stratagene). To make expression plasmids for glutathion S-transferase (GST) fusion proteins of Sam68, Sam68p21, Rev and hnRNP K, the DNA fragments were amplified by PCR and cloned into pGEX-4T-1 vector (Pharmacia). Expression plasmids pcSam68, pcSam68p21, pEGFP-Sam68 and pCMV128 and were previously described (Westberg *et al.*, 2000; Reddy *et al.*, 1999). The pc-K plasmid expressing the full-length hnRNP K and Δ 56-CT3 reporter plasmid were kindly provided by Dr G Dreyfuss (Howard Hughes Medical Institute, University of Pennsylvania) and Dr D Levens (National Cancer Institute, NIH), respectively.

Yeast two-hybrid screen

pAS2 Sam68 (292–443) was used as bait to screen a human placenta cDNA library following the Matchmaker Two-Hybrid System protocol (Clontech). Isolation, verification, and identification were done as described previously (Yang *et al.*, 1999a; Westberg *et al.*, 2000).

Cell culture, transfection

COS-1, HeLa and 293 T cells were grown at 37°C in Dulbecco's modified Eagle's medium (DMEM) with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, 50 U/ml penicillin, and 50 mg/ml streptomycin. Cells were transfected by FuGeneTM 6 Transfection Reagent (Roche) according to the manufacturer's recommendation.

Recombinant proteins

Plasmids encoding GST fusion proteins were transformed in *E. coli* BL21 following induction with isopropyl- β -D-thiogalactoside (IPTG). Recombinant GST fusion proteins were purified by incubating the bacterial extracts in buffer A (50 mM Tris pH 7.5, 100 mM NaCl, 1 mM EDTA, 1 mM DDT, 0.1 mM PMSF, 0.25% NP-40 and protease inhibitors) with glutathione-Sepharose beads (Pharmacia Biotech). The beads were then pelleted, washed five times with ice-cold buffer A and suspended in 1 ml of buffer A. GST and GST fusion proteins for RNA gel shift were eluted with buffer containing 20 mM glutathione. The purity of GST fusion proteins were examined by 10% SDS-polyacrylamide gel electrophoresis (SDS–PAGE) and stained using Coomassie Brilliant Blue. The quantity of proteins was measured by the DC protein assay (Bio-Rad).

In vitro binding assay

³⁵S-methionine-labeled hnRNP K and luciferase were synthesized by using the TNT T7/SP6 wheat germ extract-coupled system (Promega, Madison, WI, USA) according to the manufacturer's protocols. In Figure 1b and Figure 6, extracts of HeLa and 293T-cells transfected with FLAG-tagged hnRNP K were used as the protein sources, respectively. The binding assays were done as previously described (Yang *et al.*, 1999b). After extensive washes with cold binding buffer, the bound GST fusion proteins were eluted by boiling for 3 min in SDS-buffer and resolved by 10% SDS-PAGE. Western blotting was performed by a standard technique. The protein bands were visualized by enhanced chemiluminescence (Santa Cruz).

Co-immunoprecipitation assay

293T cells (6-well dish) were co-transfected with Flag or Flag-hnRNP K vector together with Sam68 expression vectors (3 µg each). Forty-eight hours later, cell extracts were prepared by lysing the cells in 1 ml of 0.8% NP-40 lysis buffer (150 mM NaCl, 10 mM Tris-HCl, pH 7.8, 1.5 mM MgCl₂, 0.8% NP-40). The resultant lysates were mixed with 40 µl of protein-A and -G agarose beads plus 10 µl of anti-Flag IgG (Stratagene). After incubating at 4°C overnight, the beads were washed three times with lysis buffer and immunoprecipitates were suspended in SDS buffer, boiled for 5 min and analysed by Western blot using anti-Sam68 antibody. To assess the expression of hnRNP K the same blot was stripped and performed Western blot analysis using the anti-Flag antibodies.

RNA gel shift assays

³²P-UTP-labeled RRE was synthesized by *in vitro* transcription with T7 RNA polymerase according to the protocols (Promega). RNA-protein binding reactions were carried out at room temperature in a total volume of 15 µl binding buffer containing 12 mM HEPES (pH 7.9), 50 mM NaCl, 4 mM MgCl₂, 10 mM DDT, 2 µg tRNA, 20 units of RNasin and 10% glycerol. Typically 1 × 10⁴ c.p.m. of ³²P-labeled RNA and 100 ng of protein were used. The binding reaction was allowed to proceed for 15 min at room temperature and the mixture was then electrophoresis on a 4.5% non-

denaturing polyacrylamide gel and visualized by autoradiography.

Microscopic examination

HeLa cells were cultured in 4-well chamber slides and co-transfected with plasmids expressing FLAG-tagged hnRNP K, GFP-Sam68 or GFP-Rev fusion proteins. Twenty-four hours post-transfection, cells were treated with 5 µg/ml actinomycin D for 3 h and subsequently stained (Yang *et al.*, 1999a). Briefly, cells were fixed for 15 min in 4% paraformaldehyde and then permeabilized by 0.5% triton X-100/PBS for 20 min at room temperature. They were then incubated with mouse monoclonal anti-FLAG antibody (Stratagene) for 1 h at 37°C. After washing with PBS, the cells were incubated with FITC-conjugated goat anti-mouse IgG antibody for 30 min at 37°C. The intracellular localization of the proteins was determined by fluorescence microscopy.

CAT assays

293T cells were cultured in 12-well plates and were transfected using FuGeneTM 6 Transfection Reagent. A β-galactosidase expression plasmid, pCDNA-LacZ, was used as an internal control for transfection efficiency. pCDNA3 was used to equalize the amount of DNA for each transfection. The cell extracts were prepared 48 h post-transfection and tested for CAT activity as described previously (Westberg *et al.*, 2000). The β-galactosidase activity was tested through standard assay procedures (Sambrook *et al.*, 1989).

p24 antigen capture assay

293T cell were co-transfected with HIV gagpol-CTE reporter plasmid pSV-gagpol-MPMVCTE, and Sam68 or hnRNP K expression plasmids. Forty-eight hours post-transfection, cell free supernatants were collected and subjected to p24 antigen assay (Coulter).

Acknowledgements

This work was supported by NIH grant GM05089 and a grant from the University Wide AIDS Research Program to F Wong-Staal, and AI46240 to TR Reddy.

References

- Argraves WS, Tran H, Burgess WH and Dickerson K. (1990). *J. Cell. Biol.*, **111**, 3155–3164.
- Bomsztyk K, Van Seuning I, Suzuki H, Denisenko O and Ostrowski J. (1997). *FEBS Lett.*, **403**, 113–115.
- Bray M, Prasad S, Dubay JW, Hunter E, Jeang KT, Rekosh D and Hammarskjöld ML. (1994). *Proc. Natl. Acad. Sci. USA*, **91**, 1256–1260.
- Chen T, Damaj BB, Herrera C, Lasko P and Richard S. (1997). *Mol. Cell Biol.*, **17**, 5707–5718.
- Chen T, Boisvert FM, Bazett-Jones DP and Richard S. (1999). *Mol. Biol. Cell*, **10**, 3015–3033.
- Denisenko ON, O'Neill B, Ostrowski J, Van Seuning I and Bomsztyk K. (1996). *J. Biol. Chem.*, **271**, 27701–27706.
- Fumagalli S, Totty NF, Hsuan JJ and Courtneidge SA. (1994). *Nature*, **368**, 871–874.
- Fusaki N, Iwamatsu A, Iwashima M and Fujisawa J. (1997). *J. Biol. Chem.*, **272**, 6214–6219.
- Hartmann AM, Nayler O, Schwaiger FW, Obermeier A and Stamm S. (1999). *Mol. Biol. Cell*, **10**, 3909–3926.
- Huang Y, Wimler KM and Carmichael GG. (1999). *EMBO J.*, **18**, 1642–1652.
- Ishidate T, Yoshihara S, Kawasaki Y, Roy BC, Toyoshima K and Akiyama T. (1997). *FEBS Lett.*, **409**, 237–241.
- Jones AR and Schedl T. (1995). *Genes Dev.*, **9**, 1491–1504.
- Lee MH, Mori S and Raychaudhuri P. (1996). *J. Biol. Chem.*, **271**, 3420–3427.
- Lin Q, Taylor SJ and Shalloway D. (1997). *J. Biol. Chem.*, **272**, 27274–27280.
- Matunis MJ, Michael WM and Dreyfuss G. (1992). *Mol. Cell Biol.*, **12**, 164–171.
- McBride AE, Taylor SJ, Shalloway D and Kirkegaard K. (1998). *Exp. Cell Res.*, **241**, 84–95.
- Michael WM, Eder PS and Dreyfuss G. (1997). *EMBO J.*, **16**, 3587–3598.

- Michelotti EF, Michelotti GA, Aronsohn AI and Levens D. (1996). *Mol. Cell. Biol.*, **16**, 2350–2360.
- Miau LH, Chang CJ, Shen BJ, Tsai WH and Lee SC. (1998). *J. Biol. Chem.*, **273**, 10784–10791.
- Reddy TR, Xu WD, Mau JK, Goodwin CD, Suhasini M, Tang H, Frimpong K, Rose DW and Wong-Staal F. (1999). *Nature Med.*, **5**, 635–642.
- Reddy TR, Xu WD and Wong-Staal F. (2000a). *Oncogene*, **19**, 4071–4074.
- Reddy TR. (2000). *Oncogene*, **19**, 3110–3114.
- Reddy TR, Tang H, Xu WD and Wong-Staal F. (2000b). *Oncogene*, **19**, 3570–3575.
- Reich E, Franklin RM, Shatkin AJ and Tatum EE. (1962). *Proc. Natl. Acad. Sci. USA*, **48**, 1238–1244.
- Renz A and Fackelmayer FO. (1996). *Nucleic Acids Res.*, **24**, 843–849.
- Resnick RJ, Taylor SJ, Lin Q and Shalloway D. (1997). *Oncogene*, **15**, 1247–1253.
- Richard S, Yu D, Blumer KJ, Hausladen D, Olszowy MW, Connelly PA and Shaw AS. (1995). *Mol. Cell. Biol.*, **15**, 186–197.
- Sambrook J, Fritsch EF and Maniatis T. (1989). *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbour, NY: Cold Spring Harbor Laboratory Press,.
- Shnyreva M, Schullery DS, Suzuki H, Higaki Y and Bomsztyk K. (2000). *J. Biol. Chem.*, **275**, 15498–15503.
- Siomi H, Matunis MJ, Michael WM and Dreyfuss G. (1993). *Nucleic Acids Res.*, **21**, 1193–1198.
- Siomi H, Choi M, Siomi MC, Nussbaum RL and Dreyfuss G. (1994). *Cell*, **77**, 33–39.
- Tomonaga T and Levens D. (1995). *J. Biol. Chem.*, **270**, 4875–4881.
- Taylor SJ, Anafi M, Pawson T and Shalloway D. (1995). *J. Biol. Chem.*, **270**, 10120–10124.
- Taylor SJ and Shalloway D. (1994). *Nature*, **368**, 867–871.
- Van Seuning I, Ostrowski J and Bomsztyk K. (1995). *Biochemistry*, **34**, 5644–5650.
- Weighardt F, Cobianchi F, Cartegni L, Chiodi I, Villa A, Riva S and Biamonti G. (1999). *J. Cell Sci.*, **112**, 1465–1476.
- Westberg C, Yang J-P, Tang H, Reddy TR and Wong-Staal F. (2000). *J. Biol. Chem.*, **275**, 21396–21401.
- Yang J-P, Hori M, Takahashi N, Kawabe T, Kato H and Okamoto T. (1999a). *Oncogene*, **18**, 5177–5186.
- Yang J-P, Hori M, Sanda T and Okamoto T. (1999b). *J. Biol. Chem.*, **274**, 15662–15670.