Mapping the Functional Domains of HAP95, a Protein That Binds RNA Helicase A and Activates the Constitutive Transport Element of Type D Retroviruses*

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The complex retroviruses such as human immunodeficiency virus, type 1, employ a virally encoded protein, Rev, to mediate the nuclear export of unspliced and partially spliced mRNA. In contrast, the simian type D retroviruses act through a cis-acting constitutive transport element (CTE) that presumably interacts directly with cellular export proteins. We first reported that RNA helicase A (RHA) is a shuttle protein that binds to functional CTE in vitro and in vivo. Recently, we isolated a novel protein, HAP95, that specifically binds to the nuclear transport domain of RHA and up-regulates CTE-mediated gene expression. Here, using truncation and deletion mutations, we mapped the domains of HAP95 that are important for RHA binding, transactivation of CTE, and nuclear cytoplasmic shuttling. We report evidence for a novel nuclear export signal in HAP95 and showed that the domains involved in RHA binding and nuclear localization are required for CTE activation. Finally, we showed that HAP95 synergizes significantly with RHA on CTE-mediated reporter gene expression and promotes nuclear export of unspliced mRNA in transfected cells. Taken together, these data support the proposal that HAP95 specifically facilitates CTE-mediated gene expression by directly binding to RHA.

Post-transcriptional regulation is an essential phase in the life cycle of many retroviruses. A key regulatory step in this phase involves the nuclear export of unspliced viral RNA, which requires the interaction of viral RNA and/or proteins with cellular factors. At least two distinct pathways have been utilized. The complex retroviruses, such as HIV,1 encode the viral protein Rev, which acts as an adapter between the Rev response element (RRE) on the viral RNA and the cellular export receptor CRM-1 (reviewed in Refs. 1 and 2). In contrast, the simian type D retroviruses do not encode a Rev-like protein but rather act through a cis-acting constitutive transport element (CTE). CTE is functionally equivalent to the Rev/RRE of HIV and presumably interacts directly with cellular export proteins to mediate the nuclear export of unspliced viral RNA. Two cellular proteins, RNA helicase A (RHA) and Tip-associated protein (3, 4), were shown to bind to functional CTE and are implicated in CTE-mediated RNA nuclear export and gene expression.

RHA was identified previously as a nuclear protein capable of unwinding RNA duplexes in an ATP-dependent manner (5). Our laboratory first reported that RHA binds specifically to functional CTE and is required for CTE activity (3). We further showed that RHA is a nuclear shuttle protein, with overlapping import and export signals localized to the carboxyl-terminal region (6). In addition, we showed that RHA plays a role in RRE-mediated gene expression (7). We proposed that RHA is required to release both CTE- and RRE-containing mRNA from spliceosomes before completion of splicing, thus freeing them for nuclear export (6, 7). Others have reported that RHA mediates molecular interactions between RNA polymerase II and the cAMP-response element-binding protein (6). In both cases, the RNA helicase synergizes with RHA in CTE-mediated gene expression (8). These reports suggest that RHA is a multifunctional protein involved in both transcriptional and post-transcriptional events.

The nuclear export of cellular mRNA and retroviral mRNA may proceed by a similar mechanism, facilitated by distinct specific RNA-binding proteins containing nuclear export signals (NESs). As part of our effort to further understand the role of RHA in post-transcriptional regulation of gene expression, we searched for functionally relevant RHA-binding proteins. A novel shuttle protein, HAP95, was identified to interact with the nuclear transport domain of RHA in the yeast two-hybrid assay (9). HAP95 also up-regulates CTE function when co-expressed with a CTE reporter gene (9). HAP95 has extensive homology with AKAP95, a member of the A-kinase anchoring protein family (10). However, HAP95 lacks the characteristic protein kinase A binding domain of this family.

In this report, we further characterize the different domains of HAP95 that may contribute to its activity on CTE-mediated gene expression. We identified a novel export signal of 17 amino acids that bears no homology with any known signal sequences, as well as two RHA-binding regions in HAP95. We also found that the amino-terminal domain of HAP95, including a YG-rich sequence, was essential for transactivation of CTE, whereas a mutant truncated in the amino-terminal region exerted a dominant negative effect. Interestingly, HAP95 synergizes with RHA on CTE-mediated gene expression in human cells. These results broaden our understanding of nu

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§ The abbreviations used are: HIV, human immunodeficiency virus; RRE, Rev response element; CTE, constitutive transport element; RHA, RNA helicase A; NES, nuclear export signal; PCR, polymerase chain reaction; GST, glutathione S-transferase; CAT, chloramphenicol acetyltransferase; PAGE, polyacrylamide gel electrophoresis; NTD, nuclear transport domain; RPA, ribonuclease protection assay; mRHA, mutant RHA.
cleo-cyttoplasmic protein transport through the identification of a new type of shunting signal and transport pathway.

**EXPERIMENTAL PROCEDURES**

**Construction of Plasmids**—The plasmids were constructed by standard methods. For generation of FLAG-tagged mutants of HAP95, the DNA fragments were amplified by PCR and cloned in-frame into pCMV-FLAG vectors (Stratagene). To make expression plasmids for HAP95 and RHA glutathione S-transferase (GST) fusion proteins, the DNA fragments were amplified by PCR and cloned into pGEX-4T-1 vector (Amersham Pharmacia Biotech). Plasmids GST-RHA-NTD and NPc-T-NLS were described previously (6). To create NPc-T-NLS-HAP95 mutants, plasmid pc-HAP95 was used as template in a series of PCRs. The individual fragments were subcloned in frame into digested NPc-T-NLS.

**pCMV138/CTE and pSV-gag-pol-MPMVCTE (gagpol-CTE)** (11, 12) were transfected by incubating the bacterial extracts in buffer A (50 mM Tris, pH 7.5, 100 mM NaCl, 1 mM EDTA, 10% glycerol). The plasmids were constructed by standard recommendations. 293T cells were grown at 37 °C in Dulbecco's modified Eagle medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamate, 50 units/ml penicillin, and 50 mg/ml streptomycin. The cells were transfected by FuGene6 Transfection Reagent (Roche Molecular Biochemicals) according to the manufacturer's recommendations. 293T cells were transfected with various FLAG-tagged HAP95 mutant expression plasmids. The cells were collected and lysed in lysis buffer (50 mM Tris, pH 7.5, 250 mM NaCl, 5 mM EDTA, 0.5 mM phenylmethylsulfonyl flouride, 0.25% Nonidet P-40, and protease inhibitors) with 10% heat-inactivated fetal bovine serum, 2 mM glutamate, 50 units/ml penicillin, and 50 mg/ml streptomycin. The quantity of proteins was measured by the DC protein assay (Bio-Rad).

**Mapping and Characterization of HAP95 Functional Domains**

**RESULTS**

Identification of a Novel NES—HAP95 was first identified as a protein that binds to the nuclear transport domain of RHA in yeast two-hybrid screen. HAP95 is partially homologous to AKAP95, a member of the protein kinase A anchoring protein family, but lacks the protein kinase A-binding domain characteristic of this family. Therefore, HAP95 is by definition not a member of the AKAP family (Fig. 1). Additionally, there are several interesting motifs in the nonhomologous regions that are unique to HAP95. In the amino terminus, there is a region with both tandem and non tandem repeats of a YG dipeptide sequence of unknown significance. In the central region, there are three nucleoporin-like FG repeats, a classical nuclear lo...
Mapping and Characterization of HAP95 Functional Domains

Fig. 2. Mapping the nuclear export signal of HAP95. A, HeLa cells were transfected with expression vectors encoding the FLAG-tagged HAP95 mutants. After expression of the transfected DNAs, the cells were fused with NIH3T3 cells to form heterokaryons and incubated in the medium containing 100 μg/ml cycloheximide for a period of 1 h. The cells were then fixed and stained for immunofluorescence microscopy with FLAG antibody to localize the proteins, and Hoechst 33258 (middle column), which differentiates the human and mouse nuclei within the heterokaryon. The arrows identify the mouse nuclei. B, summary of results depicted in A. C, definition of the minimal NES sequence. Expression vectors encoding the indicated Npc fusion proteins were transfected into HeLa cells. The heterokaryon assay was carried out as described in A. The immunofluorescence staining was carried out with mAb 9E10 antibody. A summary of the results is shown. D, the minimally defined nuclear export domain.

alization signal, and a bipartite nuclear targeting motif. The carboxyl terminus is rich in acidic residues and contains two proline-rich domains that fit the consensus for SH3-binding domains.

Although HAP95 localizes predominantly to the nucleoplasm at steady state, we previously showed that it continuously shuttles between the nucleus and cytoplasm (9). To determine the domain(s) for nuclear import and export, we carried out an interspecies heterokaryon assay (6, 9). We made constructs expressing various deletion mutants of HAP95 tagged by a FLAG epitope at their amino termini. HeLa cells were transfected with these expression plasmids and were fused to NIH 3T3 cells to form heterokaryons. Cycloheximide was added to inhibit de novo protein synthesis. The mouse nuclei were distinguished from human nuclei by their punctate staining with Hoechst 33258. As shown in Fig. 2A, the full-length as well as all the truncated mutants of HAP95 tested, except HAP1–268, were able to shuttle from human nuclei to mouse nuclei, indicating that the NLS/NES sequences reside between amino acids 268 and 349 (Fig. 2B). This segment of HAP95 is highly hydrophobic and contains two interesting repeats (EGTA and EEGKED) of unknown function.

To further isolate the NES, we fused various subfragments within the amino acids 268–384 region to a construct Npc-TNLS expressing the core domain of nucleoplasmin. SV40 large T NLS was included in these constructs to ensure complete nuclear localization of the resultant fusion proteins. As summarized in Fig. 2C, the fusion protein containing amino acid 268–384, but not the parental nonfusion protein, was exported from the nuclei of the transfected human cells. The smallest fragment sufficient to maintain NES activity contains amino acids 280–296 (Fig. 2D). Removal of five amino acid residues from the carboxyl terminus or three residues from the amino terminus of this region severely inactivated the NES (Fig. 2C). It should be noted that the HAP95 NLS was necessary for complete nuclear retention of the resultant fusion proteins. When the NLS was deleted, half of the fusion proteins containing amino acids 280–296 were found in the cytoplasm, indicating strong nuclear export activity of the NES contained therein.

Mapping the Functional Domains of HAP95—We previously showed that HAP95 is a positive co-factor for CTE function. Overexpression of HAP95 significantly increased CTE activity in transfected 293T cells (9). To further characterize the functional domains of HAP95, we made systematic deletion mutants of HAP95, each tagged by a FLAG epitope at the amino terminus (Fig. 3A), and co-transfected these with a CTE-dependent CAT reporter plasmid pCMV138/CTE into 293T cells. As shown in Fig. 3B, introduction of full-length HAP95 resulted in about a 3-fold activation in CTE function, consistent with our previous results. The amino terminus of HAP95 encompassing amino acids 1–384 was sufficient to exert full transcription activating function, suggesting that the zinc finger domain and the proline-rich domain are dispensable for activity (Fig. 3B). Consistent with this, the carboxyl-terminal fragment (amino acids 268–646) did not show any activity. Further truncation to 1–349, deleting the putative bipartite nuclear localization motif, greatly impaired the activation. Removal of the classical NLS completely abolished activity. To ensure that the variation in CTE-dependent CAT activities conferred by the HAP95 mutants was not due to differences in expression level of these
proteins, we performed a Western analysis on the cell lysates. All of the HAP95 mutants were in fact expressed at comparable levels (data not shown). These data suggest that the amino-terminal half of HAP95 is sufficient for full CTE activation.

We also determined the subcellular distribution of the various HAP95 mutant proteins after transient transfection into HeLa cells, using antibodies to the FLAG epitope. As shown in Fig. 3C, wild-type HAP95 was distributed within the nucleoplasmic region as well as concentrated in punctate loci in the nucleus but was excluded from the nucleoli. HAP95 (1–384) had the similar subcellular localization. However, when the classical NLS motif was deleted, the proteins completely localized in the cytoplasm, indicating that the bipartite nuclear motif alone was not sufficient to counter the export activity of the NES.

To map the minimal domain for HAP95 CTE function, we made additional mutations within the 1–384 fragment (Fig. 4A). As shown in Fig. 4B, deletion of both FG and NES domains only slightly reduced reporter gene expression, consistent with results with either deletion introduced into the complete HAP95 gene (data not shown). In contrast, deletion of the YG domain in this fragment or in the context of the complete HAP95 gene (data not shown) completely abolished activity. Furthermore, we found that deletion of both H1 and FG domains or of the amino-terminal 267 amino acids resulted in complete loss of CTE activation. These results suggest that the minimal region of HAP95 for CTE activation is the amino-terminal half containing the YG, H1, and NLS domains, whereas the FG and NES regions are dispensable.
HAP95 Does Not Bind to CTE RNA—We previously demonstrated that HAP95, RHA, and CTE were present in the same complex in vivo. To further support the proposal that HAP95 mediates CTE function through direct binding to RHA, we examined the possibility of HAP95 directly binding to CTE RNA. Recombinant GST and GST-HAP1–384 proteins were purified from transformed E. coli and incubated with [32P]UTP-labeled CTE RNA in binding buffer containing an excess of yeast tRNA as a nonspecific competitor. The reaction mixtures were then analyzed by the RNA gel mobility shift assay on 4.5% nondenaturing polyacrylamide gels. As shown in Fig. 5, neither GST (lane 2) nor GST-HAP1–384 (lane 3) bound to CTE RNA. In contrast, the amino terminus (1–384) of RHA, containing the two double-stranded RNA-binding domains, bound to CTE RNA (Fig. 5, lanes 4–7). Conversely, the carboxyl terminus (1014–1279) of RHA, which is sufficient for binding to HAP95, was not able to bind to CTE RNA. These data are consistent with the conclusion that CTE activation by HAP95 is mediated through its direct binding with RHA.

Functional Interaction between HAP95 and RHA—The above results prompted us to explore the functional interaction between HAP95 and RHA in vivo. We co-transfected HAP95, RHA, or their mutant expression plasmids with a CTE-dependent CAT reporter plasmid and measured CAT activity at 48 h post-transfection (Fig. 6A). HAP95 and RHA each activated CTE-dependent CAT gene expression 3–5-fold over the control, as expected. mtRHA lacking helicase activity (8), HAP95 (268–646), and AKAP95 all had little or no activation on CTE. Significantly, co-expression RHA and HAP95 synergistically increased the CAT activation to about 15-fold. This was not seen when either HAP95 (268–646) or mtRHA was used. In fact, HAP95 (268–646) completely abolished RHA-mediated CTE function, and conversely, mtRHA inhibited HAP95 activation of CTE, further supporting the functional interaction of RHA and HAP95.

Similar results were obtained with a second CTE reporter system, i.e. pSV-gagpol-MPMVCTE (11). Co-transfecting RHA or HAP95 with the reporter plasmid resulted in a 14- and 16-fold increase in gag expression as measured by p24 antigen capture assay, respectively (Fig. 6B). Again, RHA and HAP95 together exerted a synergistic effect, resulting in a 600-fold increase in p24 gag expression above the basal level. The HAP95 (268–646) mutant again had little effect on p24 gag expression on its own and significantly inhibited RHA activation in this assay.
The Rev protein of HIV-1 promotes the nuclear export of RRE-containing mRNA by directly binding to the export receptor hCRM1 through its NES (15–17). Rev function is inhibited by the antibiotic leptomycin B, which disrupts the interaction of hCRM1 and RanGTP, thus abolishing the export of the NES-containing proteins. The CTE of type D retroviruses, in contrast, functions independently of hCRM1 and is therefore insensitive to leptomycin B. This latter export pathway appears to be shared by most cellular mRNAs. Our laboratory has shown that RHA not only binds to CTE but also plays a critical role in mediating the nuclear export of CTE-containing mRNA (7). The shuttling function of RHA was not affected by leptomycin B (6). In searching for proteins that bind specifically to the NTD of RHA, we identified HAP95, which also up-regulates CTE-mediated gene expression (9). In this work, we have extended this earlier study by mapping the functional domains of HAP95 and providing strong evidence for a direct role of HAP95 in RHA-mediated nuclear export of CTE-containing mRNA.

HAP95 also shuttles between the nucleus and the cytoplasm. We identified a strong NES with 17 amino acids localized between a classical basic NLS and a bipartite NLS in HAP95 (Fig. 1). This NES has no similarity to known signals for nuclear export, including the leucine-rich NES found in Rev and the bi-directional transport signals found in hnRNP A1, hnRNP K, and RHA (6, 18, 19). Consistent with this, the HAP95 NES could not rescue the function of an NES-defective Rev mutant (data not shown). Interestingly, we found that the NES signal in HAP95 was neither necessary nor sufficient for its activation of CTE-mediated gene expression (Fig. 3B). In fact, deletion of NES resulted in a greater than 2-fold increase in activation over full-length HAP95. It is possible that the NES of RHA and HAP95 compete for a subset of cellular factors even though they may have different conditions for nucleocytoplasmic trafficking. This is evidenced by the fact that with actinomycin D treatment, RHA distributes to the cytoplasm, whereas HAP95 stays in the nucleus (data not shown). Nonetheless, further investigation on the HAP95 NES may reveal a new pathway of nuclear export for other proteins containing a similar NES.

We have mapped the minimal functional domains of HAP95 to its amino-terminal half of the protein. The critical regions include a YG-clustered region at the amino terminus, the first AKAP95 homology domain (H1), and the classical NLS (Fig. 3B). Deletion of the bipartite NLS significantly impaired but did not abolish CTE activation. Of these regions, the H1 domain appears to be required for binding of the functional subfragment HAP95 (1–384) to RHA (Fig. 4B), and NES is required for nuclear localization of the protein (Fig. 3C). Deletion of the YG domain both in full-length and (1–384) of HAP95 completely abolished their CTE activation function (Figs. 3B and 4B), without affecting their ability to shuttle or to bind RHA. Therefore, the role of this domain remains to be determined. It is intriguing to note that these YG repeats are not found in AKAP95 (Fig. 1). Despite extensive BLAST searches, we could not find similar YG repeats in other proteins. Taking together, the amino terminus of HAP95 is responsible for its activation of the CTE-mediated gene expression pathway.

The results with the H1 deletion strongly suggest that HAP95 activation of CTE requires the direct binding of RHA (Fig. 4). This conclusion was further supported by the observation that HAP95 (1–384), which was fully active, did not bind to CTE RNA (Figs. 3B and 5). Furthermore, HAP95 and RHA interaction is independent of RNA (RNase-resistant). However, the most convincing and most striking evidence for the func-

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**FIG. 7.** HAP95 increases CTE-dependent nuclear RNA export.

A, structures of the plasmid construct. The solid boxes and the thin lines denote the exon and the intron sequences from the HIV-1 gene, respectively. The CTE sequence and the HIV-1 polyadenylation sequence are also marked. The probe and the predicted protected bands are indicated. 5'ss, 5' splice site; 3'ss, 3' splice site; nt, nucleotides. B, levels of expression of the spliced (S) or unspliced (U) RNA encoded by pCMV138/CTE in the nucleus (N) or cytoplasm (C) were determined using RNase protection analysis in COS-1 cells co-transfected pCMV138/CTE in the indicated plasmids. Lanes 1 and 2, yeast RNA as control (Con.) without or with RNase. Ten micrograms of RNA was used in each lane.

To address whether the dominant negative effect of the HAP95 (268–646) is through blocking the complex formation between RHA and CTE or forming a nonproductive ternary complex, we carried out an in vivo co-immunoprecipitation assay. 293T cells were transfected with plasmid expressing CTE RNA, along with plasmids expressing FLAG-tagged HAP95, HAP95 (268–646), HAP95 (433–646), or pFLAG vector. The tagged proteins were immunoprecipitated by anti-FLAG antibodies, and the presence of CTE was verified by reverse transcription-PCR with CTE-specific primers (9). As shown in Fig. 6C, CTE RNA co-immunoprecipitated with both HAP95 and HAP95 (268–646). These results suggest that HAP95 (268–646) was still able to form a ternary complex with CTE RNA in vivo, presumably through interaction with RHA.

**HAP95 Increases CTE-dependent Nuclear RNA Export**—To explore the underlying mechanism of HAP95 and RHA on CTE-mediated gene expression, we conducted an RNase protection assay using RNA prepared from COS-1 cells transfected with pCMV138/CTE in the presence or the absence of co-expressed HAP95 or RHA. The indicator plasmid pCMV138/CTE encodes a cat reporter gene that is downstream of a functional CTE located between 5′ and 3′ splice sites (Fig. 7A). Total RNA from the nuclear and cytoplasmic fractions of COS-1 cells were isolated 48 h post-transfection, and intracellular RNA distributions were measured by RNase protection assays. As shown in Fig. 7B (lanes 3 and 4), the unspliced mRNA was detected mainly in the nucleus. However, overexpression of HAP95 or RHA dramatically increased the unspliced reporter mRNA species (lanes 6 and 8) in the cytoplasm but had no effect on the relative levels of spliced and unspliced RNA in the nuclear fraction. These results support the proposal that both RHA and HAP95 facilitated the nuclear export of unspliced, CTE-containing mRNA in human cells.

**DISCUSSION**

The Rev protein of HIV-1 promotes the nuclear export of RRE-containing mRNA by directly binding to the export receptor hCRM1 through its NES (15–17). Rev function is inhibited by the antibiotic leptomycin B, which disrupts the interaction of hCRM1 and RanGTP, thus abolishing the export of the NES-containing proteins. The CTE of type D retroviruses, in contrast, functions independently of hCRM1 and is therefore insensitive to leptomycin B. This latter export pathway appears to be shared by most cellular mRNAs. Our laboratory has shown that RHA not only binds to CTE but also plays a critical role in mediating the nuclear export of CTE-containing mRNA (7). The shuttling function of RHA was not affected by leptomycin B (6). In searching for proteins that bind specifically to the NTD of RHA, we identified HAP95, which also up-regulates CTE-mediated gene expression (9). In this work, we have extended this earlier study by mapping the functional domains of HAP95 and providing strong evidence for a direct role of HAP95 in RHA-mediated nuclear export of CTE-containing mRNA.

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tional interaction of HAP95 and RHA was their dramatic synergy on activation of CTE-mediated gene expression in vivo (Fig. 6, A and B). This synergy was specific because AKAP95, which has extensive homology with HAP95, had no such effect. An RHA mutant lacking helicase activity also failed to act on its own or to synergize with HAP95 in activating CTE-dependent gene expression.

We also directly demonstrated that both RHA and HAP95 promote an increase in cytoplasmic accumulation of CTE-containing RNA, using an RNase protection assay (Fig. 7). Because HAP95 does not directly bind CTE and its NES is not required for this effect, its exact mechanism of action is not known. We hypothesized that this effect is mediated through its direct interaction with RHA. Currently, a number of cellular proteins have been implicated in CTE-mediated gene expression. In addition to RHA and HAP95, Tap was shown to bind CTE and mediate CTE function in quail cells (20, 21). We recently reported that Sam68, which activates basal as well as Rev-mediated RRE- gene expression, also significantly activated gene expression of a CTE-gag/pol construct (13, 22). We further showed that RHA functionally interacts with Tap and Sam68 (22). Based on these and other results, we proposed that Sam68, RHA, and Tap cooperate in the post-transcriptional regulation of HIV and type D retroviral mRNA. Recently, an NTF2-like protein, p15, was found to bind to Tap and to enhance the binding of Tap to CTE (23). One can expect that additional factors relevant to this regulatory pathway will still be discovered and that these various cellular factors may contribute to the function of CTE and RRE at various steps between RNA transcription and nuclear export. These may include splicing, mRNA stability, polyadenylation, and nuclear retention. Additional insights into these processes should be gained as the network of interacting proteins and RNA is elucidated.

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