A Novel Shuttle Protein Binds to RNA Helicase A and Activates the Retroviral Constitutive Transport Element*

Received for publication, December 14, 1999, and in revised form, March 26, 2000
Published, JBC Papers in Press, March 27, 2000, DOI 10.1074/jbc.M909887199

Christopher Westberg†, Jian-Ping Yang‡, Hengli Tang, T. R. Reddy, and Flossie Wong-Staal§
From the Departments of Biology and Medicine, University of California, San Diego, La Jolla, California 92093-0665

The constitutive transport element (CTE) of type D retroviruses mediates the nuclear export of unspliced viral transcripts. We previously showed that RNA helicase A functionally interacts with CTE and contains a bidirectional nuclear transport domain at the carboxyl terminus. Here we report the identification of a novel human protein, helicase A-binding protein 95 (HAP95), which specifically binds to the carboxyl terminus of RNA helicase A. HAP95 is partially homologous to AKAP95, a member of the A kinase-anchoring protein family, but lacks the protein kinase A binding domain characteristic of this family. HAP95 is a nuclear protein at steady state but shuttles between the nucleus and cytoplasm. Overexpression of HAP95 significantly increases CTE-dependent gene expression but has no effect on general gene expression or that mediated by the Rev/Rev response element of human immunodeficiency virus type 1.

While only fully spliced cellular mRNAs are exported from the nucleus to the cytoplasm, replication of retroviruses requires the nuclear export of partially spliced and unspliced viral RNA transcripts. These transcripts serve as templates for the synthesis of a subset of viral proteins and as genomes for progeny viral particles. The complex retroviruses, exemplified by human immunodeficiency virus type 1, employ a special, virally encoded protein to mediate this export process (for a recent review, see Ref. 1). The Rev protein of human immunodeficiency virus type 1 binds to its cognate viral RNA response element, RRE, and the export receptor CRM-1 to form an active export complex (2). In contrast, the simian type D retroviruses do not encode a Rev-like protein but rather act through a cis-acting RNA element termed the constitutive transport element (CTE)† (3, 4). Thus, CTE is functionally equivalent to the Rev/RRE of human immunodeficiency virus type 1. However, the export receptor for CTE is not known.

We recently showed that RNA helicase A (RHA) specifically binds to functional CTE RNA (5) and is required for CTE activity (6). RHA was identified previously as a nuclear protein capable of unwinding RNA duplexes in an ATP-dependent manner (7). We first observed that RHA shuttles between the nucleus and cytoplasm despite its predominant nuclear localization at steady state (5) and subsequently mapped a bidirectional nuclear transport domain at the carboxyl terminus (CTD) of the protein (8). A second CTE-binding protein, TAP, has also been identified and implicated in CTE-mediated RNA nuclear export and gene expression (9, 10).

The evidence that RHA plays a role in post-transcriptional regulation of gene expression prompted us to search for RHA-interacting proteins that might also be involved in this process. Here we report the isolation of a novel human protein, named helicase A-binding protein 95 (HAP95), found by virtue of its binding to the CTD of RHA in a yeast two-hybrid screening of a human cDNA library. HAP95 has extensive homology with AKAP95, a member of the A-kinase-anchoring protein family. However, HAP95 lacks the characteristic protein kinase A binding domain of this family. Further analyses confirmed the specific interaction between RHA and HAP95 in vivo and in vitro and between HAP95 and CTE RNA in vivo. Additionally, we demonstrated that HAP95 is a shuttle protein, and overexpression of HAP95 specifically enhances CTE-mediated gene expression.

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid Screenings—The sequence corresponding to the CTD of RHA (nt 1150–1259) was amplified with PCR and cloned into the vector pGB9, in phase with the DNA binding domain of the yeast GAL4 gene. This vector was transformed into the yeast strain H7C5, along with the human leukocyte cDNA library contained in the GAL4 activation domain-expressing plasmid, pGAD10, as described previously (CLONTECH, Palo Alto, CA). Isolation, verification, and identification of plasmid clones that code for RHA-binding protein peptides was done as described previously (11).

Cloning of Additional Coding Sequence—A cDNA library carried in λ phage (agt11 from CLONTECH, human T cell lymphoma cell line, HL1089b) was plated with the Escherichia coli host strain Y1090, and nitrocellulose filter lifts (NitroBind, Micron Separations, Inc.) were made. For hybridization to these filters, a probe was created via random primed oligonucleotide synthesis (Roche Molecular Biochemicals kit) using PCR-amplified cDNA sequence from the yeast two-hybrid assay positive clone pGAD10–35 and [32P]dCTP. Positive plaques were identified through autoradiography and were isolated from primary screening plates. Two additional rounds of plating and filter hybridization were done to ensure clonality of the isolated phage. Phage DNA was isolated from large scale cultures with a Qiagen λ phage DNA purification kit and cDNA sequences were PCR-amplified from these DNA samples. Additional sequence was also obtained via PCR amplification of a human phagomid cDNA library with sequence-specific primers and high fidelity Taq polymerase from the Qiagen Hot Star kit. Sequences from the λ and phagomid libraries were cloned into carrier plasmids (pCDNA3 and pGEM-T-Easy, respectively), sequenced and later assembled into one continuous coding sequence in pCDNA3.

Cell Culture—HeLa and 293T cells were grown at 37 °C in Dulbecco’s
modified Eagle’s medium with 10% heat-inactivated fetal bovine serum, 2 mM glutamate, 50 units/ml penicillin, and 50 μg/ml streptomycin.

Northern Blot—Total RNA was prepared from tissue culture cells (grown under the conditions described above) with TRIZOL reagent (Life Technologies, Inc.) as instructed by the manufacturer and blotted onto a GeneScreen Plus membrane after calcine treatment and washing in 4°C agarose gel. Probe was prepared from clone 35 as above and hybridized to membrane as described previously (12). Ethidium-derived cell lines used were HeLa/C4 (surface marker CD4-expressing HeLa), 293, 293T (293 cells that express large T antigen of SV40), SK-n, n2, NT2, and SW13; lymphoblast-derived cell lines were H9 and Molt 4/8; the monoclonal cell line was U-937.

Recombinant Proteins—The plasmid pGEX-4T-RA-CTD, encoding glutathione S-transferase (GST) fusion protein, was transformed in E. coli strain BL21 (DE3) plyS3 following induction with 0.1 mM isopropyl-1-thio-β-D-galactopyranoside at 28 °C overnight. Recombinant GST fusion proteins were purified by incubating the bacterial extracts in buffer A (50 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol, 1 mM EDTA, 0.1 mM EGTA, 100 mM NaCl, 1 mM benzamidine, 0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM tosylphenylalanyl chloromethyl ketone, 0.25% (v/v) Nonidet P-40) with glutathione-Sepharose beads (Amersham Pharmacia Biotech). The beads were then pelleted, washed five times with ice-cold buffer A, and suspended in 1 ml of buffer B. Bound GST fusion proteins were eluted by boiling for 3 min in SDS-buffer and examined by 5–20% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) followed by staining with Coomassie Brilliant Blue to determine purity.

In Vivo Binding Assays—[35S]Methionine-labeled HAP95 and Luciferase were synthesized by using the TNT T7/SP6 wheat germ extract-coupled system (Promega, Madison, WI) according to the manufacturer’s protocols. For in vitro protein-protein interaction studies, an equal amount of the in vitro translated [35S]methionine-labeled HAP95 was incubated with 5 μg of purified GST-RA-CTD fusion protein or GST alone (as a negative control) that was bound to glutathione-Sepharose beads at 25 °C for 1 h in 250 μl of buffer A. The beads were then washed by resuspension and centrifugation five times with 1 ml of ice-cold binding buffer A. Bound proteins were eluted with an equal volume of SDS loading buffer, boiled for 3 min, resolved by 5–20% SDS-PAGE, and visualized by autoradiography.

In Vivo Binding Assays—After transfection, HeLa cells were cultured for 24 h and then harvested. After washing with PBS, cells were lysed in 350 μl of ice-cold lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 0.25% (v/v) Nonidet P-40, and protease inhibitors) for 30 min. The lysate was cleared by centrifugation. The supernatants were incubated with anti-FLAG M2 murine monoclonal antibody (Stratagene) or control mouse anti-GST monoclonal (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) overnight at 4 °C and then with protein A-Sepharose (Amersham Pharmacia Biotech) for 4 h. The beads were washed six times with 1 ml of lysis buffer. Bound proteins were eluted with an equal volume of SDS loading buffer, resolved on 10% SDS-PAGE, and visualized by autoradiography.

Co-immunoprecipitation of CTE RNA and HAP95—293T cells were transfected with plasmids expressing CTE RNA or its antisense sequence along with expression plasmids for FLAG-tagged HAP95 or Myc-tagged TAP. Cells were washed with PBS and incubated on ice with lysis buffer (150 mM NaCl, 10 mM Tris, pH 7.8, 1.5 mM MgCl₂, 20 units/ml RNasin (Promega)). Supernatants were rotated overnight at 4 °C with rabbit serum-agarose (Sigma) and then rotated overnight at 4 °C with Protein A/G Plus-agarose (Santa Cruz Biotechnology) in addition to either anti-FLAG M2 antibody (Stratagene) or anti-Myc antibody (Babco). After washing with lysis buffer, RNA was isolated from the beads with RNeasy columns (Qiagen) and treated with Dnase I. RT-PCRs (Superscript One-step system, Life Technologies, Inc.) were done with CTE-specific primers and were followed by PCRs with the same primers.

Microscopic Examination—HeLa cells were cultured in four-well chamber slides and transfected with plasmids expressing pFLAG-HAP95 fusion protein using SuperFect transfection reagent (Qiagen). After 24 h, cells were fixed with 15% formaldehyde in PBS for 45 min, then permeabilized with 0.5% Triton X-100/PBS for 20 min at room temperature. They were then incubated with mouse monoclonal anti-FLAG antibody for 1 h at 37 °C. After washing with PBS, the cells were incubated with fluorescein isothiocyanate-conjugated goat anti-mouse IgG antibody for 30 min at 37 °C.

Heterokaryon Assay—HeLa cells were transfected with a plasmid pGFP-HAP95 expressing the HAP95 protein fused to green fluorescence protein. The formation of heterokaryons from NIH3T3 and HeLa cells as well as subsequent cycloheximide treatment, cell fusion, fixation, and staining, were done as described previously (8, 13).

Chloroprophilic Acetyltransferase (CAT) Assays—293T cells were transfected with plasmids expressing pCAT 3′-CAT-3′-CAT containing the CAT gene along with either CTE or RRE within splice donor sites (pDM138-CTE and pDM128) were transfected (5). The expression vector for HAP95 was also transfected in some experiments. Transfactions with the RRE-CAT reporter were done with Rev expression plasmid. To test the effects of HAP95 on gene expression that is not dependent on retroviral elements, the plasmid pCAT-3′ control (Promega) was used. A β-galactosidase plasmid was used as an internal control of transfection efficiency. Leptomycin B (4 nm) was used in some experiments. Transfected cells were washed with 1× PBS and fed fresh medium after 12 h. Cells were harvested 48 h post-transfection. Cell extracts were prepared and tested for CAT activity through standard assay procedures as described previously (12).

RESULTS

Molecular Cloning and Characterization of a Novel RNA Helicase A-Binding Protein—To identify proteins that could potentially interact with RHA in vivo and play a role in nuclear export, we performed yeast two-hybrid screening of a human leukocyte cDNA library, using the carboxyl terminus of RHA as bait. After several rounds of transformant screening, seven independent clones identifying six different putative RHA binding proteins were identified. Two of these had no significant homology with characterized proteins in the GenBank™ database. One of these novel clones, number 35, had a contiguous open reading frame of 893 nt throughout the cDNA segment of the plasmid. We concluded that this sequence probably represents the internal coding sequence of a larger gene. Additional sequences at both the 5′- and 3′-ends of the gene were obtained by screening a Agt11 phage library with a radioactive probe derived from clone 35 cDNA. About 1 kilobase of sequences were obtained 5′ of clone 35 cDNA, but these constituted mostly untranslated sequences. The 180 nucleotides 3′ of clone 35 cDNA extend the open reading frame but still did not contain any termination codons. For the remaining 3′ coding sequence, we fortuitously found a GenBank™ entry of a 883-nt sequence (GenBank™ accession no. AF053596), the first 257 nt of which were an exact match to the last 257 nt of the 3′ cDNA clone. This entry was subsequently retracted, but nonetheless, based on its information, we were able to construct a 3′-specific primer to amplify our target sequence from a phagemid library. Amplification with nested primers and sequencing were used to confirm the identity of the amplified sequence.

The coding sequences from the two λ clones and the amplified sequence from the phagemid library were assembled into a single gene (Fig. 1A) and cloned into pcDNA3. Nucleotide sequence analysis revealed in-frame stop codons in the 5′-untranslated region followed by two potential initiating ATG codons. In comparison of the predicted in vitro translated proteins (homology products from intact and truncated constructs identified the second ATG as the true initiating codon (data not shown). These data predict a 72-kDa, 648-amino acid protein, but its migration on SDS-PAGE yielded an apparent size of approximately 100 kDa (data not shown). Search of the GenBank™ data base revealed a high degree of homology between the novel protein and AKAP95, a member of the A-kinase-anchoring protein family (14). In deference to this homology, we dubbed the novel protein HAP95 (helicase A-binding protein 95) (GenBank™ accession no. AF199414). The homology between HAP95 and AKAP95 is distributed in two distinct areas, one of which contains two zinc-finger DNA binding motifs (Fig. 1B). However, it should be noted that HAP95 is not a member of the AKAP family, since it lacks the characteristic amphi-
Novel RHA-binding and CTE-activating Shuttle Protein

A

1.

το\,y\,ν\,σ\,τ\,ι\,ν\,β\,α\,κ\,ρ\,ι\,ν\,τ\,ι\,ν\,α\,π\,ι\,ν\,τ\,ι\,ν\,α\,κ\,ρ\,ι\,ν\,τ\,ι\,ν\,α\,κ\,ρ\,ι\,ν\,τ\,ι\,ν\,α\,κ\,ρ\,ι\,ν\,τ\,ι\,ν\,α\,κ\,ρ\,ι\,ν\,τ\,ι\,ν\,α\,κ\,ρ\,ι\,ν\,τ\,ι\,ν\,α\,κ\,ρ\,ι\,ν\,τ\,ι\,ν\,α\,κ\,ρ\,ι\,ν\,τ\,ι\,ν\,α\,κ\,ρ\,ι\,ν\,τ\,ι\,ν\,α\,κ\,ρ\,ι\,ν\,τ\,ι\,ν\,α\,κ\,ρ\,ι\,ν\,τ\,ι\,ν\,α\,κ\,ρ\,ι\,ν\,τ\,ι\,ν\,α\,κ\,ρ\,ι\,ν\,τ\,ι\,ν\,α\,κ\,ρ\,ι\,ν\,τ\,ι\,ν\,α\,κ\,ρ\,ι\,ν\,τ\,ι\,ν\,α\,κ\,ρ\,ι\,ν\,τ\,ι\,ν\,α\,κ\,ρ\,ι\,ν\,τ\,ι\,ν\,α\,κ\,ρ\,ι\,ν\,τ\,ι\,ν\,α\,κ\,ρ\,ι\,ν\,τ\,ι\,ν\,α\,κ\,ρ\,ι\,ν\,τ\,ι\,ν\,α\,κ\,ρ\,ι\,ν\,τ\,ι\,ν\,α\,κ\,ρ\,ι\,ν\,τ\,ι\,ν\,α\,κ\,ρ\,ι\,ν\,τ\,ι\,ν\,α\,κ\,ρ\,ι\,ν\,τ\,ι\,ν\,α\,κ\,ρ\,ι\,ν\,τ\,ι\,ν\,α\,κ\,ρ\,ι\,ν\,τ\,ι\,ν\,α\,κ\,ρ\,ι\,ν\,τ\,ι\,ν\,α\,κ\,ρ\,ι\,ν\,τ\,ι\,ν\,α\,κ\,ρ\,ι\,ν\,τ\,ι\,ν\,α\,κ\,ρ\,ι\,ν\,τ\,ι\,ν\,α\,κ\,ρ\,ι\,ν\,τ\,ι\,ν\,α\,κ\,ρ\,ι\,ν\,τ\,ι\,ν\,α\,κ\,ρ\,ι\,ν\,τ\,ι\,ν\,α\,κ\,ρ\,ι\,ν\,τ\,ι\,ν\,α\,κ\,ρ\,ι\,ν\,τ\,ι\,ν\,α\,κ\,ρ\,ι\,ν\,τ\,ι\,ν\,α\,κ\,ρ\,ι\,ν\,τ\,ι\,ν\,α\,κ\,ρ\,ι\,ν\,τ\,ι\,ν\,α\,κ\,ρ\,ι\,ν\,τ\,ι\,ν\,α\,κ\,ρ\,ι\,ν\,τ\,ι\,ν\,α\,κ\,ρ\,ι\,ν\,τ\,ι\,ν\,α\,κ\,ρ\,ι\,ν\,τ\,ι\,ν\,α\,κ\,ρ\,ι\,ν\,τ\,ι\,ν\,α\,κ\,ρ\,ι\,ν\,τ\,ι\,ν\,α\,κ\,ρ\,ι\,ν\,τ\,ι\,ν\,α\,κ\,ρ\,ι\,ν\,τ\,ι\,ν\,α\,κ\,ρ\,ι\,ν\,τ\,ι\,ν\,α\,κ\,ρ\,ι\,ν\,τ\,ι\,ν\,α\,κ\,ρ\,ι\,ν\,τ\,ι\,ν\,α\,κ\,ρ\,ι\,ν\,τ\,ι\,ν\,α\,κ\,ρ\,ι\,ν\,τ\,ι\,ν\,α\,κ\,ρ\,ι\,ν\,τ\,ι\,ν\,α\,κ\,ρ\,ι\,ν\,τ\,ι\,ν\,α\,κ\,ρ\,ι\,ν\,τ\,ι\,ν\,α\,κ\,ρ\,ι\,ν\,τ\,ι\,ν\,α\,κ\,ρ\,ι\,ν\,τ\,ι\,ν\,α\,κ\,ρ\,ι\,ν\,τ\,ι\,ν\,α\,κ\,ρ\,ι\,ν\,τ\,ι\,ν\,α\,κ\,ρ\,ι\,ν\,τ\,ι\,ν\,α\,κ\,ρ\,ι\,ν\,τ\,ι\,ν\,α\,κ\,ρ\,ι\,ν\,τ\,ι\,ν\,α\,κ\,ρ\,ι\,ν\,τ\,ι\,ν\,α\,κ\,ρ\,ι\,ν\,τ\,ι\,ν\,α\,κ\,ρ\,ι\,ν\,τ\,ι\,ν\,α\,κ\,ρ\,ι\,ν\,τ\,ι\,ν\,α\,κ\,ρ\,ι\,ν\,τ\,ι\,ν\,α\,κ\,ρ\,ι\,ν\,τ\,ι\,ν\,α\,κ\,ρ\,ι\,ν\,τ\,ι\,ν\,α\,κ\,ρ\,ι\,ν\,τ\,ι\,ν\,α\,κ\,ρ\,ι\,ν\,τ\,ι\,ν\,α\,κ\,ρ\,ι\,ν\,τ\,ι\,ν\,α\,κ\,ρ\,ι\,ν\,τ\,ι\,ν\,α\,κ\,ρ\,ι\,ν\,τ\,ι\,ν\,α\,κ\,ρ\,ι\,ν\,τ\,ι\,ν\,α\,κ\,ρ\,ι\,ν\,τ\,ι\,ν\,α\,κ\,ρ\,ι\,ν\,τ\,ι\,ν\,α\,κ\,ρ\,ι\,ν\,τ\,ι\,ν\,α\,κ\,ρ\,ι\,ν\,τ\,ι\,ν\,α\,κ\,ρ\,ι\,ν\,τ\,ι\,ν\,α\,κ\,ρ\,ι\,ν\,τ\,ι\,ν\,α\,κ\,ρ\,ι\,ν\,τ\,ι\,ν\,α\,κ\,ρ\,ι\,ν\,τ\,ι\,ν\,α\,κ\,ρ\,ι\,ν\,τ\,ι\,ν\,α\,κ\,ρ\,ι\,ν\,τ\,ι\,ν\,α\,κ\,ρ\,ι\,ν\,τ\,ι\,ν\,α\,κ\,ρ\,ι\,ν\,τ\,ι\,ν\,α\,κ\,ρ\,ι\,ν\,τ\,ι\,ν\,α\,κ\,ρ\,ι\,ν\,τ\,ι\,ν\,α\,κ\,ρ\,ι\,ν\,τ\,ι\,ν\,α\,κ\,ρ\,ι\,ν\,τ\,ι\,ν\,α\,κ\,ρ\,ι\,ν\,τ\,ι\,ν\,α\,κ\,ρ\,ι\,ν\,τ\,ι\,ν\,α\,κ\,ρ\,ι\,ν\,τ\,ι\,ν\,α\,κ\,ρ\,ι\,ν\,τ\,ι\,ν\,α\,κ\,ρ\,ι\,ν\,τ\,ι\,ν\,α\,κ\,ρ\,ι\,ν\,τ\,ι\,ν\,α\,κ\,ρ\,ι\,ν\,τ\,ι\,ν\,α\,κ\,ρ\,ι\,ν\,τ\,ι\,ν\,α\,κ\,ρ\,ι\,ν\,τ\,ι\,ν\,α\,κ\,ρ\,ι\,ν\,τ\,ι\,ν\,α\,κ\,ρ\,ι\,ν\,τ\,ι\,ν\,α\,κ\,ρ\,ι\,ν\,τ\,ι\,ν\,α\,κ\,ρ\,ι\,ν\,τ\,ι\,ν\,α\,κ\,ρ\,ι\,ν\,τ\,ι\,ν\,α\,κ\,ρ\,ι\,ν\,τ\,ι\,ν\,α\,κ\,ρ\,ι\,ν\,τ\,ι\,ν\,α\,κ\,ρ\,ι\,ν\,τ\,ι\,ν\,α\,κ\,ρ\,ι\,ν\,τ\,ι\,ν\,α\,κ\,ρ\,ι\,ν\,τ\,ι\,ν\,α\,κ\,ρ\,ι\,ν\,τ\,ι\,ν\,α\,κ\,ρ\,ι\,ν\,τ\,ι\,ν\,α\, κριν\,τιν\,α\,π\,ε\,ν\,τ\,ι\,ν"}
a HAP95 cDNA probe revealed ubiquitous expression of two mRNA species of 2.0 and 4.0 kilobases, respectively (Fig. 2).

Interaction of HAP95 with RHA and CTE RNA—The carboxyl terminus of RHA (RHA-CTD) was expressed as a GST-tagged fusion protein. The purified GST-RHA-CTD fusion protein, bound to glutathione-Sepharose beads, was incubated with 35S-labeled HAP95 or luciferase (control) and washed extensively, and the eluted material was analyzed by SDS-PAGE and autoradiography. As shown in Fig. 3a, HAP95 interacted strongly with RHA (lane 3). This binding appears to be specific, since no interaction was seen between HAP95 and the GST moiety alone (lane 2) or between luciferase and GST-RHA-CTD or GST (lanes 5 and 6).

In order to examine whether RHA binds to HAP95 in vivo, HeLa cells were transfected with either the plasmid expressing FLAG-tagged HAP95 (pFLAG-HAP95) or the pFLAG vector. Cell extracts from these transfected cells were immunoprecipitated with either murine monoclonal anti-FLAG or anti-GST antibodies. The immunoprecipitates were fractionated by SDS-PAGE and blotted with rabbit polyclonal anti-RHA antibodies. As shown in Fig. 3b, RHA was co-immunoprecipitated with FLAG-HAP95 by anti-FLAG antibodies in the transfected cells (lane 5). Immunoprecipitates made with a control anti-GST antibody did not demonstrate a strong immunoreactive RHA band (lane 3), and no RHA bands were seen in immunoprecipitates made from cells overexpressing the FLAG epitope by itself (lanes 2 and 4). Preliminary data indicate that HAP95 did not bind to TAP or to Sam68, two other RNA binding proteins involved in post-transcriptional regulation of retroviral mRNA (16, 17) (data not shown).

To demonstrate the interaction of CTE RNA and HAP95 in vivo, 293T cells were transfected with plasmids that express RNA containing the CTE sequence or its antisense, along with pFLAG-HAP95 or Myc-tagged TAP, a known CTE-binding protein. The tagged proteins were immunoprecipitated by virtue of their peptide tags, and the presence of CTE or antisense CTE RNA was verified by RT-PCR and subsequent PCR amplification with CTE-specific primers. As shown in Fig. 4, CTE RNA, but not the antisense sequence, immunoprecipitated with HAP95 and TAP. As a control, RT-PCR was performed on total RNA without immunoprecipitation from transfected cells, which verified that both CTE constructs are expressed in similar amounts in vivo (data not shown).

HAP95 Is a Nuclear Shuttle Protein—To elucidate the subcellular localization of HAP95, we transfected HeLa cells with pFLAG-HAP95. Staining with the anti-FLAG antibodies revealed the fusion protein to be localized within the nucleoplasmic region as well as concentrated in punctate loci in the nucleus but excluded from the nucleoli (Fig. 5a). Heterokaryon assays were then performed to determine if HAP95 is able to shuttle between the nucleus and the cytoplasm. HeLa cells were transfected with a plasmid (pGFP-HAP95) expressing HAP95 fused to green fluorescent protein. After 24 h, protein synthesis was halted by treatment with cycloheximide, and the cells were fused to NIH-3T3 to form interspecies heterokaryons as described previously (8, 13). Three hours after fusion, cells were fixed and examined by fluorescence microscopy. The mouse nuclei were distinguished from human nuclei by punctate staining with Hoechst 33258. Green fluorescence was observed in both human and mouse nuclei within the same heterokaryons, demonstrating that HAP95 had been exported from the HeLa cell nuclei and imported into the NIH-3T3 cell nuclei (Fig. 5b). Thus, HAP95 is primarily localized to the nucleus at steady state but is able to continuously shuttle between the nucleus and the cytoplasm.
did not show any activity (data not shown). Additional negative controls include human immunodeficiency virus LTR-CAT and CRE (cyclic AMP-responsive element)-CAT (data not shown). The up-regulation of CTE-dependent gene expression by HAP95 was dose-dependent (data not shown). Like the basal CTE-CAT activity, activation of CTE-CAT by HAP95 was not inhibited by leptomycin B, in contrast to Rev-mediated transactivation of RRE-CAT (Fig. 6).

**DISCUSSION**

Retroviruses utilize at least two distinct pathways to export unspliced mRNA from the nucleus to the cytoplasm. The complex retroviruses, such as human immunodeficiency virus, encode a viral protein Rev, which acts as an adaptor between the RRE RNA element and the export receptor, CRM1 (2, 18). In contrast, simian type D retroviruses utilize a cis-acting constitutive transport element (CTE), which presumably interacts directly with cellular export proteins (3, 4). The export receptor for this pathway is not known, but it is distinct from CRM-1. Two CTE-binding cellular proteins have been identified. TAP, the human homolog of the yeast protein Mex67, was shown to bind specifically to CTE and enhance nuclear export of CTE in the *Xenopus* oocyte system (9). This is consistent with the findings that Mex67 is involved in yeast mRNA nuclear export (19, 20). Furthermore, TAP was recently shown to be able to partially rescue CTE function in nonpermissive cell lines (10, 16). Our laboratory reported that RHA binds specifically to functional CTE (5), and antibodies to RHA strongly inhibited CTE activity when microinjected into cell nuclei (6). We further showed that RHA is a nuclear shuttle protein, with overlapping import and export signals localized to the CTD. The export function of RHA is also independent of CRM-1 (8), consistent with the hypothesis that RHA may play a role in CTE export. Interestingly, RHA also seems to be involved in RRE-mediated gene expression at a step upstream of export (6).

In an effort to identify other cellular proteins that may be involved in CTE export and/or function, we searched for proteins that bind RHA, initially using the yeast two-hybrid screening assay. We used only the CTD of RHA instead of the...
Novel RHA-binding and CTE-activating Shuttle Protein

entire 140-kDa protein as a bait, since this region contains the bidirectional nuclear transport domain. Here we report the identification of a novel protein, HAP95, which was confirmed to bind RHA both in vivo and in vitro. HAP95 does not bind to TAP, the other CTE-binding protein. Interestingly, HAP95 shows significant homology to AKAP95, a member of the A-kinase-anchoring protein family. The AKAP family bind the regulatory subunits of protein kinase A (PKA) and localize PKA to various distinct locations and structures within cells. This subcellular localization is believed to be crucial for the appropriate function of PKA in cellular signaling pathways. The binding of PKA regulatory subunits is mediated by a characteristic amphipathic helix in AKAP (reviewed in Ref. 21). AKAP95, specifically, binds the RIα subunit of PKA with high affinity (22). In addition to this amphipathic peptide region, it also contains two zinc finger DNA-binding domains in its carboxyl-terminal half of the protein. Alignment of HAP95 to AKAP95 revealed two regions of homology separated by nonhomologous sequences. The homologous region closer to the amino terminus (residues 91–232 of HAP95, 31% identity and 71% similarity with AKAP95) contains the two nonhomologous sequences. The homologous region closer to the amino terminus (residues 91–232 of HAP95, 31% identity and 47% similarity with AKAP95) has no defined function. The second homologous region (residues 385–547 of HAP1, 51% identity and 71% similarity with AKAP95) contains the two zinc finger DNA-binding motifs. Notably, the amphipathic helix in AKAP95 responsible for binding to the RIα subunit of PKA is absent in HAP95. Thus, HAP95 by definition is not a member of the AKAP family. Conversely, HAP95 has several features not found in AKAP95. At the amino terminus, there is a region with many YG dipeptide motifs of unknown significance. The central region of the protein contains a basic stretch that could serve as a nuclear localization signal as well as an RNA binding domain. There are also three nucleoporin-like FG repeats in this region (23). The carboxyl terminus is rich in acidic residues and contains two proline-rich domains that fit the consensus for SH3 binding domains (15). Search of the EST database uncovered sequences derived from HAP95. Interestingly, HAP95 and AKAP95 appear to both reside on human chromosome 19 (GenBank™ accession numbers AC005785 and AC006128), suggesting that HAP95 could have arisen from AKAP95 through gene duplication.

Using a heterokaryon assay, we found that a GFP-HAP95 fusion protein is able to shuttle between nucleus and cytoplasm although its steady state localization is predominantly found in the nucleus. It is not clear whether HAP95 shuttling is dependent on its binding to RHA or vice versa. More importantly, we showed that HAP95 is a positive co-factor for CTE function. Overexpression of HAP95 significantly increased CTE activity in transfected 293T cells. We demonstrated that HAP95 binds CTE RNA in vivo, although this may be mediated by an intermediate protein or complex, most likely involving RHA. We recently observed that RHA and TAP also directly interact with each other. The actual mechanisms by which RHA, HAP95, and TAP interact to mediate CTE export and expression remain to be elucidated.

Acknowledgment—We thank Dr. Minoru Yoshida for the gift of leptomycin B.

Addendum—While this manuscript was in press, Orstavik et al. (24) published on the cloning and characterization of a novel protein, HA95, which is identical to HAP95.

REFERENCES

1. Hope, T. J. (1999) Arch. Biochem. Biophys. 365, 186–191
2. Fornerod, M., Ohno, M., Yoshida, M., and Mattaj, I. W. (1997) Cell 90, 1051–1060
3. Bray, M., Prasad, S., Duhay, J. W., Hunter, E., Jeang, K. T., Rekosh, D., and Dreyfuss, G. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 1256–1260
4. Zolotukhin, A. S., Valentine, A., Pavlakis, G. N., and Felber, B. K. (1994) J. Virol. 68, 7944–7952
5. Tang, H., Gaietta, G. M., Fischer, W. H., Ellisman, M. H., and Wong-Staal, F. (1997) Science 276, 1412–1415
6. Li, J., Tang, H., Mullin, T., Westberg, C., Reddy, T. R., Rose, D. W., and Wong-Staal, F. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 709–714
7. Lee, C. G., and Hurwitz J. (1992) J. Biol. Chem. 267, 4598–4607
8. Tang, H., McDonald, D., Middleworth, T., Hope, T. J., and Wong-Staal, F. (1999) Mol. Cell. Biol. 19, 3540–3550
9. Gruter, P., Tahernero, C., Von Kobbe, C., Schmitt, C., Saavedra, C., Bachi, A., Wilm, M., Felber, B. K., and Izaurralde, E. (1998) Mol. Cell 1, 649–659
10. Kang, Y., and Cullen, B. R. (1999) Oncogene 14, 2785–2792
11. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd ed., pp. 7.37, 7.39, 7.52, 16.59, and 16.63, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
12. Michael, W. M., Choi, M., and Dreyfuss, G. (1995) Cell 83, 415–422
13. Eide, T., Coghlan, V., Orstavik, S., Solberg, R., Skalhegg, B. S., Lamb, N. J., Coghlan, V. M., Langeberg, L., Fernandez, A., Scott, J. D., Jahnsen, T., and Tasken, K. (1996) Exp. Cell Res. 228, 305–316
14. Feller, S. M., Ren, R., Hanafusa, H., and Baltimore, D. (1994) Trends Biochem. Sci. 19, 453–458
15. Bear, J., Tan, W., Zolotukhin, A., Tahernero, C., Hudson, E. A., and Felber, B. K. (1999) Mol. Cell Biol. 19, 6306–6317
16. Reddy, T. R., Xu, W., Mau, J. K. L., Goodwin, C. D., Suhasini, M., Tang, H., Frimpong, K., Rose, D. W., and Wong-Staal, F. (1999) Nat. Med. 5, 635–642
17. Farjot, G., Sergeant, A., and Mikaelian, I. (1999) J. Biol. Chem. 274, 17309–17317
18. Sainos-Rosa, H., Moreno, H., Simos, G., Segref, A., Fahrenkrog, B., Pante, N., and Hurt, E. (1998) Mol. Cell. Biol. 18, 6826–6838
19. Segref, A., Sharma, K., Doye, V., Hellwig, A., Huber, J., Luehrmann, R., and Hurt, E. (1997) EMBO J. 16, 3256–3271
20. Colledge, M., and Scott, J. D. (1999) Trends Cell Biol. 9, 216–221
21. Coghlan, V. M., Langeberg, L. K., Fernandez, A., Lamb, N. J., and Scott, J. D. (1994) J. Biol. Chem. 269, 7685–7683
22. Rout, M. P., and Wente, S. R. (1994) Trends Cell Biol. 4, 357–365
23. Orstavik, S., Eide, T., Collins, P., Han, I. O., Tasken, K., Kieff, E., Jahnsen, T., and Skalhegg, B. S. (2000) Biol. Cell 92, 27–37

H. Tang and F. Wong-Staal, submitted for publication.
A Novel Shuttle Protein Binds to RNA Helicase A and Activates the Retroviral Constitutive Transport Element

Christopher Westberg, Jian-Ping Yang, Hengli Tang, T. R. Reddy and Flossie Wong-Staal

*J. Biol. Chem.* 2000, 275:21396-21401.
doi: 10.1074/jbc.M909887199 originally published online March 27, 2000

Access the most updated version of this article at doi: 10.1074/jbc.M909887199

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 23 references, 12 of which can be accessed free at http://www.jbc.org/content/275/28/21396.full.html#ref-list-1