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The p80 homology region of TEP1 is sufficient for its association with the telomerase and vault RNAs, and the vault particle

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ABSTRACT

TEP1 is a protein component of two ribonucleoprotein complexes: vaults and telomerase. The vault-associated small RNA, termed vault RNA (VR), is dependent upon TEP1 for its stable association with vaults, while the association of telomerase RNA with the telomerase complex is independent of TEP1. Both of these small RNAs have been shown to interact with amino acids 1–871 of TEP1 in an indirect yeast three-hybrid assay. To understand the determinants of TEP1–RNA binding, we generated a series of TEP1 deletions and show by yeast three-hybrid assay that the entire Tetrahymena p80 homology region of TEP1 is required for its interaction with both telomerase and VRs. This region is also sufficient to target the protein to the vault particle. Electrophoretic mobility shift assays using the recombinant TEP1 RNA-binding domain (TEP1–RBD) demonstrate that it binds RNA directly, and that telomerase and VRs compete for binding. VR binds weakly to TEP1–RBD in vitro, but mutation of VR sequences predicted to disrupt helices near its central loop enhances binding. Antisense oligonucleotide-directed RNase H digestion of endogenous VR indicates that this region is largely single stranded, suggesting that TEP1 may require access to the VR central loop for efficient binding.

INTRODUCTION

Vaults are 13 million Dalton ribonucleoprotein particles (RNPs) of unknown function with dimensions of 42 × 75 nm (1–3). Vault morphology is reminiscent of a barrel with a cap on each end and is highly conserved across a wide range of species. The basic vault structure is formed by multimerization of a predicted 96 copies of the 96 kDa major vault protein (MVP), which makes up >70% of the particle mass and forms the entire exterior shell of vaults (4). Virtually all of the MVPs of the cell biochemically fractionate as a large particle, suggesting that all or most MVP monomers are incorporated into vaults. Although the function of vaults has not been determined, roles have been proposed with respect to nucleocytoplasmic trafficking, multi-drug resistance and, more recently, as a scaffold for epidermal growth factor signaling (5–9). Despite their presence in a wide variety of evolutionarily diverse organisms and upregulation in several multi-drug resistant cancer cell lines, mammalian vaults are not required for survival, as mice lacking MVP are viable, do not display increased sensitivity to cytostatic drugs, and have no obvious phenotypic defects (10).

Vaults contain two other proteins: vault poly (ADP-ribose) polymerase (VPARP) and telomerase-associated protein 1 (TEP1), as well as a small untranslated vault RNA (VR) (11–13). Each of these components is present in multiple copies in vaults, but their precise stoichiometry with respect to a single vault is unclear, and it is not known whether all vaults contain equal numbers of these components. Moreover, some species, such as human and bullfrog, express multiple-related VRs that can associate with the vault particle (8,11). Cryoelectron microscope (cryoEM) image reconstructions of intact or RNase-treated vaults purified from rat liver have resolved the structure to 31 and 22 Å resolutions, respectively. Difference imaging between intact and RNase-treated vaults localizes VR to the interior ends of each of the vault caps (3,14). The precise localization of VPARP and TEP1 within the vault particle is not yet known. Both proteins, as well as VR, are also found in non-vault-associated fractions within the cell (8,12,13).
TEP1 is an RNA-binding protein that interacts with mammalian telomerase in cell extracts and was cloned based upon its homology with *Tetrahymena* p80, which also co-purified with telomerase activity and binds the *Tetrahymena* telomerase RNA (TR) *in vitro* (15–17). A schematic illustration of the various domains identified in TEP1 is shown in Figure 1. Disruption of the p80/p95 complex in *Tetrahymena* leads to telomere lengthening (18). However, p80 is not a core telomerase component and is not required for telomerase activity (18–20). Furthermore, recombinant p80 binds poorly or not at all to other co-expressed telomerase subunits in *Escherichia coli* or insect cell extracts and nonspecifically interacts with a number of RNAs *in vitro*, in addition to its interaction with TR (19). The precise function of mammalian TEP1 is also not known, but it has been shown to be one of four components of purified vaults and to interact with the VR and mammalian TR in an indirect yeast three-hybrid assay (13,16). Immunoprecipitation of endogenous TEP1 co-immunoprecipitates telomerase activity (16,17). Despite the *in vitro* association between TEP1 and telomerase activity, Tep1-deficient mice have no known telomerase-related defect, as telomerase activity, TR levels and telomere length are normal in these mice (21,22). However, VR levels are reduced in all tissues studied from Tep1-deficient mice and VR stability is markedly reduced in mouse embryonic fibroblasts (MEFs) derived from these mice in comparison with MEFs derived from wild-type mice. Furthermore, vaults purified from Tep1-deficient mice do not contain VR, demonstrating genetically and biochemically that TEP1 is required for the association of VR with the vault particle (22).

VPARP is a member of the PARP family of proteins and is the only vault-associated protein demonstrated to have enzymatic activity, as it is able to ADP-ribosylate both itself and MVP *in vitro*, although the functional significance of this modification is not known (12). In addition to its localization in the vault particle, VPARP is seen at the mitotic spindle using immunofluorescence, and immunoprecipitation of VPARP from lysates derived from VPARP transfected cells results in the co-immunoprecipitation of telomerase activity (12,23). Like TEP1, Vparp-deficient mice also have no known phenotypic defects, telomerase-related or otherwise (22,23). Vaults purified from livers of either Vparp- or Tep1-deficient mice also appear morphologically normal (22,23).

VR is an RNA polymerase III transcript that does not appear to be further processed, and thus retains its 3' polyuridylylate tail, binds the La autoantigen *in vitro* and is found stably complexed with La apart from the vault particle *in vivo* (24). Interestingly, La has also been found to co-purify with vaults purified from rat livers. The genes for VR have been identified and sequenced for a variety of species, and the RNAs are all predicted to fold into a highly conserved structure (8,25). Some species, such as humans and bullfrogs, express multiple-related VRs, and two species, humans and mouse, each contains a VR pseudogene (8,25,26). The function of VR is not known, but as it is unimportant for the structural integrity of the vault particle, it is proposed to play a functional rather than structural role in the complex (14,27).

The goal of the current study is to determine whether TEP1, a component of both vault and telomerase RNP s, each containing an unrelated RNA, VR or TR, binds singly or simultaneously to these RNA species. Using the yeast three-hybrid system, we determined that the entire TEP1 *Tetrahymena* p80 homology region is required for its interaction with VR and TR. Electrophoretic mobility shift assays (EMSAs) using a partially purified truncation of TEP1 containing this RNA-binding domain (TEP1–RBD) demonstrate that TEP1 can indeed bind RNA directly, and that, like p80, TEP1 also binds some RNAs non-specifically. However, although TR binds to TEP1–RBD *in vitro*, only point mutants of mVR that are predicted to disrupt base-pairing near the VR central loop were able to interact with TEP1–RBD, suggesting that other factors are normally required for this association. Furthermore, competition experiments indicate that there is likely only one RNA-binding site, since VR and TR do not simultaneously bind to TEP1–RBD. RNase H digests of extracts containing VR annealed to complementary DNA oligonucleotides (ODNs) were synthesized in order to identify single-stranded regions of VR. These experiments suggest that the central loop of partially purified endogenous VR forms a more open and single-stranded structure than predicted by thermodynamic models. Combined with the results of our

![Figure 1](image-url)
VR mutations, these results suggest that the central loop of VR is important for its interaction with TEP1. Finally, by purifying recombinant vaults generated by co-infecting SF9 insect cells with baculoviruses expressing MVP and a series of TEP1 truncations, we show that the p80 homology region of TEP1 also contains the vault-interaction domain.

**MATERIALS AND METHODS**

**Yeast three-hybrid analysis**

Mouse TEP1 deletions were generated by PCR, cloned into the pACT II yeast expression vector and confirmed by sequence analysis. PCR primers were engineered to contain an NcoI site for cloning purposes and are as follows. Forward primers for the following TEP1 amino acid sequences 1–871, 1–740 and 1–580: 5′-AAATCCATTGCTATGGAGAAGCTCTGTTGGCCATG-3′; 201–871: 5′-AAATCCATTGCTCAAAGAAAGAAAGCAC-AAGAG-3′; 270–871: 5′-AAATCCATTGCTCTGGACTTACCCGGGCATC-3′; and 400–871: 5′-AAATCCATTGCTCGCCTCCTAAGACAGAAGC-3′. Reverse primers: 1–871; 201–871; 270–871 and 400–871: 5′-AAATCCATTGCTATGGAGAAGCTCTGTTGGCCATG-3′. Mouse VR was cloned by PCR into the pMAL site of pII/MS-2 in sense and antisense orientations using the following primers: forward, 5′-CTGACTAGTACGGCCG GCCAGTCCATGAG-3′; reverse, 5′-TTGACACTGTCGTACGGCCG GCCAGTCCATGAG-3′. Mouse VR plasmids were transformed into the L40i yeast strain and tested for TEP1–VR and TEP1–TR interactions as described previously (13,16,28).

**TEP1–RBD expression and partial purification**

The DNA sequence for amino acids 201–871 of murine TEP1 was PCR amplified and cloned into pET28a using forward and reverse primers containing engineered SalI and NotI sites, respectively, thus fusing the His7-T7 tag onto the N-terminus of TEP1–RBD. Forward primer, 5′-GCTTTCTAGACTAACCCATTGCTATTGCTACGGCCG GCCAGTCCATGAG-3′; reverse primer, 5′-ATAAGAATGCGGCCGCTTTATAGTTTATC TAGTGTCCTCC-3′; 1–740: 5′-ATAATCGCGGTTCCATCACA TAAATCCTCCTC-3′; and 1–580: 5′-AAATCCATTGCTCG CAAAGAAAGAAAGCAC-AAGAG-3′. Murine TR clones for use in the yeast three-hybrid system have been published previously (16). Plasmids were transformed into the L40i yeast strain and tested for TEP1–VR and TEP1–TR interactions as described previously (13,16,28).

**RNase H digests**

RNase H assays were performed on either P100 extract (RNP) or deproteinized P100 extract (RNA) prepared from either rat fibroblasts or HeLa cells. Antisense ODNs that span almost the entire length of rat VR or human VR1 were synthesized on an Applied Biosystems DNA synthesizer. Ten micrograms of total protein (P100) or deproteinized P100 (RNA), 0.5 µg of antisense ODNs and 0.4 U of RNase H (in a final volume of 25 µl) were incubated at 30°C for 1 h. Reactions were phenol:chloroform extracted, and ethanol precipitated in the presence of 10 µg of carrier tRNA and analyzed by northern blotting as described previously (8).

**Baculoviruses, SF9 insect cell infections and vault purifications**

The rat MVP-encoding baculovirus and human TEP1-encoding baculovirus have been described previously (4,29). DNA encoding human TEP1 amino acids 1–1650 and 1–125 were generated by PCR, cloned into a pFastBac 1 and verified by sequencing. The primers used were as follows. The 1–1650 DNA encoding baculovirus have been described previously (4,29). DNA encoding human TEP1 amino acids 1–1650 and 1–125 were generated by PCR, cloned into a pFastBac 1 and verified by sequencing. The primers used were as follows. The 1–1650 DNA encoding baculovirus have been described previously (4,29). DNA encoding human TEP1 amino acids 1–1650 and 1–125 were generated by PCR, cloned into a pFastBac 1 and verified by sequencing. The primers used were as follows. The 1–1650 DNA encoding baculovirus have been described previously (4,29). DNA encoding human TEP1 amino acids 1–1650 and 1–125 were generated by PCR, cloned into a pFastBac 1 and verified by sequencing. The primers used were as follows. The 1–1650 DNA encoding baculovirus have been described previously (4,29). DNA encoding human TEP1 amino acids 1–1650 and 1–125 were generated by PCR, cloned into a pFastBac 1 and verified by sequencing. The primers used were as follows. The 1–1650 DNA encoding baculovirus have been described previously (4,29).
fluorescent dideoxy-nucleotide sequencing and automated detection (ABI/PerkinElmer). The resulting 956 amino acid fusion protein has a predicted weight of 108.4 kDa. Viruses were generated according to the protocol of the Bac-to-Bac system (Invitrogen). Sf9 cells were maintained and infected with baculovirus(es) as described previously (4). Recombinant vaults were purified from infected Sf9 cell lysates and analyzed by western blot as described previously (4).

RESULTS

The entire Tetrahymena p80 homology region of murine TEP1 is required for the interaction with vault and telomerase RNAs

A truncation consisting of the first 871 amino acids of murine TEP1 has been previously shown to interact with mouse TR and multiple human VRs using a yeast three-hybrid assay (13,16). Briefly, this assay fuses an RNA of interest to the MS2 phage hairpin RNA, which binds to the MS2 coat protein, and can be used to test RNA–protein interactions by driving transcription of a reporter gene in a manner analogous to yeast two-hybrid assays (28). In addition to the p80 region, this 871 amino acid sequence contains four N-terminal 30 amino acid repeats of unknown significance. Since the region of homology between TEP1 and p80 is poorly conserved with other RNA-binding proteins at key residues (30), we decided to test a series of TEP1 truncations for two reasons: first, to determine whether it was possible to separate the RNA-binding activities for VR and TR, which might suggest a functional connection between the two RNAs; and second, if this was not possible, to further define the region of TEP1 critical for binding RNA. TEP1 deletions were made using the 1–871 amino acid region as a starting point (Figure 1A), and these constructs were transformed into the L40i yeast strain, along with plasmids expressing mTR and mVR sense and control, antisense RNAs fused to the MS2 hairpin RNA. An interaction between protein and RNA activates the His3 reporter gene and allows the growth of yeast on media lacking histidine and containing 3-aminotriazole. Both the 1–871 amino acid construct and a construct lacking the 4 N-terminal repeats of TEP1 (amino acids 201–871) were able to bind to both TR and VR in sense but not antisense orientations based on this assay (Figure 1A, constructs 1 and 2; Figure 1B, 1s, 1as, 2s and 2as). Deletion of any sequences in the TEP1-p80 homology region resulted in the loss of RNA-binding activity of both TR and VR (Figure 1A, constructs 3–6 and Figure 1B, 3–6). Western blots of transformed yeast cultures verified that all of the TEP1 deletions were expressed (data not shown), although a substantial portion of construct 3 (amino acids 270–871) is found as a smaller breakdown product in yeast lysates. Thus, the entire p80 homology region (amino acids 201–871) is required for TEP1 to bind both VR and TR in an indirect yeast three-hybrid system; henceforth this domain will be referred to as TEP1–RBD.

Partially purified TEP1–RBD binds TR in vitro

Evidence exists that VR and TR bind to TEP1 in vitro and in vivo, but it has not yet been shown that TEP1 binds to these RNAs directly (13,16,22). Therefore, we expressed and purified from E.coli the murine TEP1–RBD fused to the HisT7 N-terminal epitope tag derived from pET28 (see Materials and Methods). We were unable to completely purify the recombinant protein away from contaminating bacterial proteins without washing TEP1–RBD off the Ni-NTA resin. Nevertheless, we were able to partially purify enough recombinant TEP1–RBD (Figure 2A) to determine whether it could interact with RNA in vitro using EMSA. Although full-length mTR binds to TEP1–RBD using EMSA (data not shown), we also transcribed mTR nt 1–223, which is sufficient to interact with TEP1 in the yeast three-hybrid assay (A. Reda and L. Harrington, unpublished data), rather than full-length mTR, to facilitate resolution of the RNA and RNP complexes on native gels. Incubation with increasing amounts of TEP1–RBD induced a small shift in the mobility of the 223 nt mTR probe using EMSA (Figure 2B, lanes 1–4), and this association was competed with pre-incubation of 50x unlabeled full-length TR (Figure 2B, lane 5). Surprisingly, even 250x excess unlabeled mVR was unable to compete with the TR probe for binding to TEP1–RBD (Figure 2B, lane 6), but an mVR variant (see below) did compete with TR probe, although less effectively than TR itself (Figure 2B, compare lanes 7–8 with lane 5). Using more than 80 ng TEP1–RBD in the binding reactions did not result in increased TR binding, nor did using more than 50-fold molar excess of unlabeled TR result in much additional competition with labeled probe (data not shown), indicating that the majority of the RNA is unable to bind despite a high molar excess of protein to target RNA. To test the specificity of RNA binding by TEP1, four non-specific competitor RNAs were used; three RNAs were unable to compete for binding to TEP1 even at 500x excess (Figure 2B, lanes 9–11). The NL15 RNA is an artificial RNA with both single- and double-stranded regions that interacts with the La protein in vivo, but does not bind in a 3' uridylic acid dependent manner (31). La has previously been shown to interact with the VR and the TR (24,32,33). As La also interacts with the vault particle, this seemed to be a relevant RNA to test for binding to TEP1–RBD (24). However, in vitro transcription of the multiple-cloning site of linearized pBluescript SK+ containing the T7 promoter yielded an RNA, which was able to efficiently compete with TR for binding at 50-fold molar excess (Figure 2B, lane 12). This indicates that TEP1–RBD, like its Tetrahymena p80 homolog, has limited specificity with respect to its affinity for RNA in vitro (19). The specificity of the RNA–protein complex was then further analyzed since the TEP1–RBD protein was only partially purified. We used a monoclonal antibody directed against the T7 epitope tag of the recombinant TEP1–RBD protein to co-immunoprecipitate the mTR (nt 1–223) probe (Figure 2C, lanes 1 and 2). Control antibodies (Figure 2C, lanes 3–6) did not bring down either the TEP1–RBD protein or the mTR RNA, demonstrating that the band shift seen using EMSA was specifically due to binding to TEP1–RBD.

A VR variant has an enhanced ability to bind TEP1–RBD in vitro

Since unlabeled mVR could not compete with TR for binding to TEP1–RBD in vitro, we repeated the EMSAs using radiolabeled VR transcripts. When labeled mVR was
incubated with TEP1–RBD, no RNP complex was observed, as might be expected from the inability of mVR to compete with labeled mTR for binding to TEP1–RBD (Figure 3A, left panel). In contrast, when we used the related human hVR1, we detected a low-level interaction with the murine TEP1–RBD using EMSA (Figure 3A, right panel). We recently reported that the mouse genome contains two VR genes, mvg1 and mvg2.

Figure 2. Partially purified murine TEP1–RBD interacts with TR in vitro but has limited specificity. (A) Amino acids 201–871 of murine TEP1 were expressed in E.coli and partially purified using the hexahistidine tag derived from the pET28a vector. Shown are a Coomassie-stained gel (left panel) and western blot (right panel) using anti-TEP1 polyclonal antibodies. (B) EMSA of nt 1–223 of mouse TR incubated with TEP1–RBD. Five fmol 32P-labeled probe was incubated with 0, 5, 20 and 80 ng TEP1–RBD (lanes 1–4). Probe was competed off with 50x full-length TR (lane 5) and increasing amounts of mVR double point mutant (lanes 7 and 8), but not wild-type mVR (lane 6). The 250x unlabeled 5S Ribosomal RNA, tRNA phe or an artificial RNA (NL15) do not compete with the probe (lanes 7–9), but 50x excess of an artificial RNA derived from the pBluescript polylinker region does compete efficiently (lane 10). Labeled RNA is indicated with a black dot and shifted complexes are indicated with an asterisk. (C) Immunoprecipitation of TEP1–RBD from binding reactions using anti-T7 monoclonal antibody co-immunoprecipitates the TR transcript. Equivalent amounts of the bound (B) and unbound (U) fraction were analyzed by either western blot using anti-TEP1 polyclonal antibody (upper panel) or by fractionation on a 10% acrylamide/8 M urea gel (lower panel). The latter gel was dried and radioactive bands visualized by phosphorimager analysis. Antibodies to the T7 epitope (lanes 1 and 2), but not antibodies to the FLAG (lanes 3 and 4) and VSVG (lanes 5 and 6) epitopes, immunoprecipitated both TEP1–RBD and TR.

Figure 3. Mutagenesis of murine VR enhances its binding to TEP1–RBD. (A) EMSA of VR–TEP1–RBD complexes. Binding reactions contained 5 fmol of 32P-labeled murine VR (left panel) or human VR1 (right panel) incubated with 0 (lanes 1 and 4), 20 (lanes 2 and 5) and 80 (lanes 3 and 6) ng of TEP1–RBD. (B) EMSA of VR probes (5 fmol) without (−) or with (+) 80 ng TEP1–RBD in the binding reaction. Wild-type mouse VR interacts poorly or not at all with TEP1–RBD (lanes 1 and 2), but a highly related sequence in the mouse genome that is not expressed binds more strongly (lanes 3 and 4). A VR double point mutant G70A, C73U (lanes 5 and 6) binds more strongly to TEP1–RBD. Also shown are the corresponding single mutants in mouse VR (lanes 7–10) and human VR1 (lanes 11 and 12). Labeled RNAs are indicated with a black dot and shifted complexes are indicated with an asterisk. (C) The thermodynamically predicted secondary structure of the wild-type (left structure) and double point mutant (center structure) mouse VR, focusing on the regions of interest only. Also shown is the entire predicted structure of mouse VR, along with the position of the double point mutant and the C78 to A mutation, which has enhanced binding.
munirine VR clone identified from our original genominc screen for VR sequences and was initially presumed to be the one and only VR gene. This VR pseudogene (henceforth termed \( \Psi mVR \)) contains only 8 nt changes as compared with mVR, and is predicted to form into the evolutionarily conserved VR structure. Thus, we had initially expressed the \( \Psi mVR \) "coding region" in vitro using a T7 promoter and determined that this VR pseudotranscript is also able to interact with TEP1–RBD in mobility shifts (Figure 3B, lane 4).

This observation provided a starting point for a mutational analysis strategy to determine why mVR was unable to bind to TEP1–RBD in vitro. \( \Psi mVR \) sequences were replaced with mVR sequences in a stepwise manner (Table 1) and assayed for the loss of binding to TEP1 using EMSA. The first column of Table 1 indicates the position of the 8 nt differences between \( \Psi mVR \) and mVR, and subsequent columns indicate intermediate mVR sequences after nucleotides were altered to revert to the mVR sequence 1 or 2 nt at a time. We were able to revert the \( \Psi mVR \) sequence back to the mVR sequence without losing binding to TEP1–RBD until reversion was attempted at either nt 70 or 73. Once the key residues responsible for \( \Psi mVR \)’s interaction with TEP1–RBD were identified, these residues were altered in mVR, and it was confirmed using EMSA that this mVR double mutant is able to interact with TEP1–RBD (Figure 3B, compare lanes 4, 6 and 12). The double mutant alters the base pairing from Watson–Crick to wobble, and is predicted to open a loop in this region of the VR (Figure 3C, left and center structures) using the mfold RNA structure prediction program (34). Because this structure borders the conserved central loop of VR (defined as the region where all three arms of the VR converge in the nine different VR sequences identified across the various species to date), we hypothesized that mutations opening up the central loop might also increase TEP1 binding. Novel mutations in unmodified mVR, which might weaken the stability of the helix adjacent to the central loop without grossly altering the predicted structure of VR, were synthesized. One mutation, C78–A, showed enhanced binding to TEP1–RBD similar to the mVR double mutant (data not shown) (results summarized in Figure 3C, right structure).

The specificity of the interaction between the mVR double mutant and TEP1–RBD was tested using a number of unlabeled competitor RNAs. Addition of increasing amounts of TEP1–RBD to the mobility shift binding reactions resulted in an increasingly intense band representing the mVR double mutant–protein complex (Figure 4, lanes 1–4). Pre-incubation with 50× excess of the unlabeled VR double mutant competed most of mobility shift, as did 50× excess TR (Figure 4, lanes 5 and 6). However, not all of the labeled RNA could be competed, similar to TR competition results (Figure 2B). This VR/TR competition for binding to TEP1–RBD indicates that it is likely that either VR or TR, but not both simultaneously, is able to bind to TEP1. Increasing the amount of TEP1–RBD to more than 80 ng did not result in increased VR binding, nor did incubation with more than 50× unlabeled VR double mutant result in additional competition with labeled probe (data not shown). The same four non-specific competitor RNAs were used as with labeled TR, with identical results; again only the SK+ transcript was able to compete for binding (Figure 4, lanes 7–10). Addition of the anti-T7 monoclonal antibody to the binding reaction also resulted in a faint supershift on EMSA (data not shown).

**Table 1. Stepwise reversion of \( \Psi mVR \) to mVR sequence**

| Nucleotide | \( \Psi mVR \) | 1 | 2 | 3 | 4 | 5 | 6 | mVR |
|-----------|--------------|---|---|---|---|---|---|-----|
| 16        | G            | G | G | G | G | G | G | C   |
| 28        | U            | U | C | C | C | C | C | C   |
| 36        | U            | U | G | G | G | G | G | C   |
| 70        | A            | A | A | A | A | A | A | G   |
| 73        | U            | U | U | U | U | C | U | C   |
| 91        | G            | G | G | G | A | A | A | C   |
| 100       | A            | A | A | A | G | G | G | C   |
| 121       | G            | G | C | C | C | C | C | C   |

The position of the 8 nt differences between the two RNAs are shown in the leftmost column, and the base found in \( \Psi mVR \) and mVR at each is indicated. Columns 1–6 are variant VRs tested for binding to TEP1–RBD, with the result shown in the bottom row.

**Figure 4.** The mVR point mutant competes with TR for binding to TEP1–RBD. EMSA of mVR double point mutant incubated with 0, 5, 20 and 80 ng of TEP1–RBD (lanes 1–4). Unlabeled mVR mutant or mouse TR (50×) competes with the labeled probe (lanes 5 and 6). Three irrelevant RNAs do not compete with TR for binding to TEP1–RBD (lanes 7–9), but the pBluescript transcript does compete efficiently (lane 10) as in Figure 2. Labeled RNA is indicated with a black dot and shifted complexes are indicated with an asterisk.

**RNase H mapping of VR indicates that regions surrounding the central loop are single stranded**

We hypothesized that *in vivo* the central loop of the VR may be in a more open conformation that may facilitate TEP1 binding. To determine whether sequences in the central loop of the VR were available for base pairing, we used an oligodeoxynucleotide-directed RNase H cleavage assay (35). We undertook a preliminary analysis of the secondary structure of both rat VR (Figure 5, left panels), which is highly related to murine VR, and human VR1 (Figure 5, right panels) using this assay. Vault-associated VR was obtained from

**Species:**
- **Human:**
  
**Species:**
- **Human:**
  
**Species:**
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resuspended high speed (100 000 g) pellets and deproteinated VR obtained from phenol:chloroform extraction of these resuspended pellets. A series of overlapping ODNs complementary to the VRs were each annealed to VR from either rat fibroblasts or HeLa cell lysates for rat and human VRs, respectively. RNase H was then added to cleave RNA–DNA hybrids, which form in regions where RNA secondary structure is largely single stranded (35). The extent of cleavage of the 141 nt rat VR was analyzed by northern blotting (Figure 5A). In the absence of VR-associated vault proteins, three ODNs, 21–40, 54–70 and 68–85, directed nearly complete cleavage of rat VR (Figure 5A, lower left panel, RNA). In the presence of vault proteins, these three ODNs again led to the most substantial cleavage of VR. According to these data, both rat and human VR have a significantly more open structure in vivo in the regions surrounding the central loop than was predicted by thermodynamic models (Figure 5B, left and right panels). We found minor differences between protein-bound and deproteinated RNA, but overall the rat and human VRs were found to be single stranded in regions of VR that are poorly conserved in sequence across species. That may indicate that it is the structure and not the sequence per se that is important for TEP1–RBD binding.

The p80 region of TEP1 also binds vaults

Although TEP1 did not interact with either MVP or VPARP in a yeast two-hybrid assay (36), TEP1 does interact directly with MVP, as co-infection of baculoviruses expressing TEP1 and MVP generates vault-like particles in insect cells that, when purified, contain TEP1 (29). Thus, TEP1 may be able to interact with intact vault particles but not MVP monomers. In order to identify a TEP1–MVP interaction domain, we generated a series of TEP1 truncations for use in the baculovirus expression system (Figure 6A). Baculoviruses expressing the various TEP1 truncations were then used to infect SF9 insect cell cultures, either alone or in combination with an MVP-expressing baculovirus. Vaults were purified from the co-infected cells as described previously (4). As a final purification step, fractions enriched in recombinant vaults were layered over a 10–60% discontinuous sucrose gradient; vaults comprising HisT7-epitope tagged MVP subunits were used in this study. These particles fractionate predominantly in the 45% sucrose layer (Figure 6B, ‘MVP’ panel). When TEP1 truncations were expressed without MVP, the vault purification scheme removed each truncation prior to the sucrose gradient step [Figure 6B, 1–3, upper (−) panels]. When expressed together with MVP [Figure 6B, 1–3, lower (+) panels], some TEP1 truncations were seen to co-purify with the recombinant vaults [Figure 6B, 1–3, lower (+) panels]. Truncations consisting of amino acids 1–1050 and 1–911 (Figure 6A, constructs 1 and 2; Figure 6B, panels 1 and 2) each co-purified with vaults, whereas a truncation consisting of amino acids 1–125, which contains the 4 N-terminal repeats, failed to interact (Figure 6A, construct 3 and Figure 6B, panel 3). By deduction, amino acids 126–911 contain a vault-interaction domain. A fourth TEP1 construct, consisting of amino acids 201–1650 of murine TEP1, also interacted with vaults even though it was poorly expressed in insect cells, possibly due to the deletion of the TEP1 N-terminus (data not shown). Therefore, the p80 region of TEP1
and 3, lower panels). To interact with vaults are found largely in the 45% sucrose layer (column 1, 2, P100, or both, and are lost in the purification scheme before the sucrose gradient fractionation, TEP1 truncations fractionate in either the high-speed supernatant (S100), sucrose layer (upper panel). In the absence (pH 6.5 and analyzed by western blot. Vaults fractionate largely in the 45% for 16 h. Sucrose fractions were pelleted, resuspended in 20 mM MES buffer, final step, samples were loaded onto a 20–60% sucrose gradient and centrifuged, acting proteins will co-purify with vaults. Vaults were further enriched, and as a (P100) of cell lysates and can be purified to near-homogeneity. Vault-interacting proteins will co-purify with vaults. Vaults were further enriched, and as a final step, samples were loaded onto a 20–60% sucrose gradient and centrifuged for 16 h. Sucrose fractions were pelleted, resuspended in 20 mM MES buffer, pH 6.5 and analyzed by western blot. Vaults fractionate largely in the 45% sucrose layer (upper panel). In the absence (−) of MVP-baculovirus co-infection, TEP1 truncations fractionate in either the high-speed supernatant (S100), P100, or both, and are lost in the purification scheme before the sucrose gradient step (1, 2, and 3, upper panels). When MVP is present (+), TEP1 truncations able to interact with vaults are found largely in the 45% sucrose layer (column 1, 2, and 3, lower panels).

Figure 6. The p80 homology region of TEP1 contains the vault interaction domain. (A) TEP1 truncations were co-expressed with the MVP in Sf9 insect cells using a baculovirus expression vector. (B) MVP forms vaults when expressed in insect cells, which are found entirely in the high-speed pellet (P100) of cell lysates and can be purified to near-homogeneity. Vault-interacting proteins will co-purify with vaults. Vaults were further enriched, and as a final step, samples were loaded onto a 20–60% sucrose gradient and centrifuged for 16 h. Sucrose fractions were pelleted, resuspended in 20 mM MES buffer, pH 6.5 and analyzed by western blot. Vaults fractionate largely in the 45% sucrose layer (upper panel). In the absence (−) of MVP-baculovirus co-infection, TEP1 truncations fractionate in either the high-speed supernatant (S100), P100, or both, and are lost in the purification scheme before the sucrose gradient step (1, 2, and 3, upper panels). When MVP is present (+), TEP1 truncations able to interact with vaults are found largely in the 45% sucrose layer (column 1, 2, and 3, lower panels).

(amino acids 201–911) appears to contain both the RNA-binding and vault-interaction domains. The VR is not present in the baculovirus expression system, and Sf9 insect cells do not contain endogenous vault components, so the TEP1–vault interaction is not mediated by VR. The mapping of a vault-interaction domain to this conserved region suggests the intriguing possibility that *Tetrahymena* might contain vaults or a protein conferring a related function. A database search of the *Tetrahymena* genome did not identify an MVP homolog, although assembly of the sequences for this genome is not yet complete (The Institute for Genomic Research website at http://www.tigr.org).

**DISCUSSION**

**PSI-blast searches using the amino acid sequence of p80 have previously identified two conserved regions that together span the entire length of the protein. First, the TROVE (Telomerase, Ro and Vault) module was identified as an evolutionarily conserved (presumed) multi-domain region found in TEP1, p80, Ro60 and a number of uncharacterized bacterial proteins (30). This region spans amino acids 227–685 of murine TEP1. Ro60 binds to small RNAs termed ‘Y RNAs’ along with the La autoantigen in a small RNP complex and has been proposed to contain an RNA recognition motif (RRM domain) based upon the presence of RNP-1 and RNP-2 motifs that are the hallmark of this domain (37,38). However, putative RRMs in p80 and TEP1 have an unusually short spacing of the RNP-1 and RNP-2 motifs and contain conserved polar residues in regions that are non-polar in other RRMs (30). The second domain found using databases searches of p80 is a von Willebrand type A (vWA) domain, which can be involved in a number of functions such as binding to metals, or mediating protein–protein interactions, often in extracellular proteins or in multiprotein complexes (39). This domain is also found adjacent to the TROVE module in TEP1, and corresponds to amino acids 678–861 (29). Interestingly, a vWA domain is found in VPARP as well (30). Our study indicates that an intact TROVE module is required for RNA-binding activity, but we were unable to remove the vWA sequences from the p80 homology region of TEP1 in our yeast three-hybrid assay without interfering with the ability of TEP1 to bind RNA. Since vWA domains are not known to bind RNA, it is possible that the removal of this region disrupts the conformation of the RNA-binding domain in the TROVE module. An analysis of Ro60 similarly determined that even small amino acid deletions in the protein led to the loss of binding to Y RNAs, so it appears that this aspect of the two proteins has also been conserved (40). As we have also mapped the vault-interaction domain of TEP1 to the p80 homology region, either the TROVE module or the vWA domain must mediate this interaction. TEP1 interacts directly with MVP in the insect cell expression system, and is not dependent upon VR for its association with vaults (29). Thus, it is not clear what mechanism targets VR but not TR into the vault particle. One possibility is that since the RNA-binding and vault-binding regions of TEP1 are either in close proximity or overlap, binding of TR to TEP1 could interfere with the ability of the protein to complex with vaults, while binding of VR to TEP1 could promote the TEP1–vault interaction. However, the effect of VR or TR on the stability of the TEP1–vault association is difficult to assess using the insect cell expression system, since the vault purification scheme incorporates an RNase treatment step to remove ribosomes.

Since both VPARP and TEP1 associate with vaults and telomerase (16,17,23), it is possible that there is a functional connection between the two RNPs. The present study indicates that VR or TR, but not both, can interact with a given TEP1 molecule, so it does not seem that VR could directly associate with the telomerase complex via TEP1 without displacing TR. Likewise, neither telomerase activity nor telomerase RNA has been found associated with purified rat liver vaults or vaults immunoprecipitated from tissue culture cells, respectively, so it does not appear likely that either RNA is to be found in the other RNP complex (13,26). The results of our RNase H experiments suggest that the *in vivo* structure of the VR diverges significantly from structure predictions based upon thermodynamic models. Specifically, the left and right arms of VR, which are essentially the only regions of the RNA that are not conserved across species or even within a single species
when multiple VRs are present, are largely single stranded. The central loop of purified endogenous VR is, therefore, considerably larger than seems thermodynamically likely, which may be due to binding of TEP1 to the conserved lower region of the loop. It also seems likely that the in vitro transcribed VR used in EMSA might fold into a structure resembling the more thermodynamically favorable predicted structures shown in Figure 5, rather than a structure containing large stretches of single-stranded RNA. This would explain why only a small fraction of the RNA interacts with TEP1–RBD in vitro. Mutations in VR that disrupt base pairing near the VR central loop enhance binding to TEP1 in vitro, which suggests that this could be the case, and that these mutations might partially compensate for the absence of factors promoting the TEP1–VR interaction in vivo. Such missing factors could include MVP, VPARP and, in particular, the La RNA-binding protein, which binds VR stably apart from the vault particle and loosely purifies with vaults, suggesting that it may promote the VR–TEP1 interaction in vaults. Additionally, the full-length TEP1 contains a putative NTPase motif, which could potentially regulate the association of TEP1 with VR. Thus far, we have been unable to purify sufficient quantities of recombinant TEP1 containing both the p80 and putative NTPase regions to test this hypothesis, since TEP1 is particularly susceptible to degradation when expressed in insect cells. Finally, it is difficult to rule out misfolding of either RNA or the TEP1–RBD protein when conducting in vitro experiments using purified components, either of which could affect the specificity and stability of the RNA–protein interaction.

It was surprising that wild-type mouse VR did not readily interact with TEP1–RBD, since human VR1, mutant mVR, TR and a non-specific RNA did interact. Genetic evidence has demonstrated that TEP1 directs VR to the vault particle and influences its stability in vivo. Therefore, this study suggests that additional components are probably required for TEP1 to bind specifically to RNA. The complexity of reconstituting the TEP1–VR interaction in vitro may explain why non-specific binding of some RNAs to TEP1 is observed in the current study, and also why studies of p80–TR binding also reported non-specific binding of RNA (19). Overall, these results indicate that binding of RNA to TEP1 may be regulated by other cellular factors and suggest that the RNA-binding properties of TEP1 in vitro are probably similar to those seen with p80.

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