The JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 277, No. 43, Issue of October 25, pp. 41282–41286, 2002
Printed in U.S.A.

The La RNA-binding Protein Interacts with the Vault RNA and Is a Vault-associated Protein*

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Vaults are highly conserved ubiquitous ribonucleoprotein particles with an undefined function. Three protein species (p240/TEP1, p193/VPARP, and p100/MVP) and a small RNA comprise the 13-MDa vault particle. The expression of the unique 100-kDa major vault protein is sufficient to form the basic vault structure. Previously, we have shown that stable association of the vault RNA with the vault particle is dependent on its interaction with the p240/TEP1 protein. To identify other proteins that interact with the vault RNA, we used a UV-cross-linking assay. We find that a portion of the vault RNA is complexed with the La autoantigen in a separate smaller ribonucleoprotein particle. La interacts with the vault RNA (both in vivo and in vitro) presumably through binding to 3'-uridylates. Moreover, we also demonstrate that the La autoantigen is the 50-kDa protein that we have previously reported as a protein that co-purifies with vaults.

Vaults are cytoplasmic ribonucleoprotein particles (RNPs)† with an unusual ovoid structure (1–3). Although the exact function of vaults is not yet known, in humans, the elevated vault levels have been used to predict whether tumors will acquire multidrug resistance (reviewed in Ref. 4). They have a mass of 13 MDa, and their three-dimensional reconstruction to 22-Å resolution indicated that the inside of the barrel is spacious enough to encompass an intact ribosome, consistent with a carrier/sequestration capacity (5, 6). Unlike ribosomes, the vault particle is formed by only a few different proteins, one of which multimerizes to constitute >70% of the particle mass. This protein, termed the major vault protein (MVP), is the main structural component of the particle because its expression alone is sufficient to induce vault particle formation (7). Two other proteins and a small RNA comprise the rest of the vault particle. These protein subunits have masses of 193 (VPARP) and 240 kDa (TEP1) (8, 9). VPARP is a functional poly(ADP-ribose) polymerase, and TEP1 is essential for the stable association of the vault RNA with the particle (10). TEP1 is a protein subunit that is shared with the telomerase RNP complex; however, its role in telomerase function is unclear (11). A vault-interacting protein of ∼50 kDa has been reported to co-purify with vaults prepared from rat liver (12).

Besides the proteins, a unique small untranslated RNA of 141 bases termed vault RNA (vRNA) is associated with rat vaults (13). The rat vRNA component is estimated to account for approximately 5% of the particle mass or 16 copies/vault particle (12). Difference mapping of the various vault reconstructions indicated that the vRNA is localized to the particle caps in proximity to TEP1 (6). The genes encoding human, rat, and mouse vRNAs have been cloned and sequenced (13–15). The vault RNA genes are transcribed by RNA polymerase III and contain the canonical A and B box internal promoter elements along with several external promoter elements (16). Some species like human and bullfrog contain multiple related vRNAs. The vertebrate vRNAs range in size from 86–141 bases in length, and despite their differences in length, they are predicted to fold into similar secondary structures. The vRNA is not a structural component of the vault particle, as its digestion with ribonucleases does not alter particle morphology (6, 17). Our current hypothesis is that vRNA is a functional component of the vault particle, possibly through the mediation of protein-RNA and/or RNA-RNA interactions.

Ultimately, our goal is to understand the function of the vault particle and the role of its associated RNA. We have undertaken a biochemical analysis of the vRNA and its association with other proteins in cellular extracts. Early characterization of vault particles revealed that vaults pelleted with the microsomal fraction at 100,000 × g (P100) (2, 12). Here we show that although most of the major vault protein is associated with the microsome fraction, a significant amount of TEP1, VPARP, and vRNA is associated with the high speed (100,000 × g) supernatant (S100) and nuclear fractions. We demonstrate that the vRNA can be UV-cross-linked with a 50-kDa protein in the S100 fraction to form a small RNP complex. We have identified this protein as the La/SS-B autoantigen (reviewed in Refs. 18–20). Partial purification of the complex demonstrates that vRNA and the La/SS-B autoantigen co-fractionate by DEAE-Sepharose chromatography. Anti-La serum specifically immunoprecipitates a portion of the cellular vRNA, and gel shift analysis shows that La specifically binds to vRNA. Finally, we identify La as the “50-kDa” protein that has been reported to co-purify with vaults isolated from rat liver (12).

EXPERIMENTAL PROCEDURES

Subcellular Fractionation and UV-cross-linking Analysis—Nuclear, S100, and P100 extracts were prepared from HeLa cells as described.

*This research was supported in part by United States Public Health Service Grant GM38097 from the National Institutes of Health and a grant from the G. Harold and Leila Y. Mathers Charitable Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1754 solely to indicate this fact.

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1 The abbreviations used are: RNP, ribonucleoprotein particle; MVP, major vault protein; VPARP, vault poly(ADP-ribose) polymerase; TEP1, telomerase-associated protein 1; vRNA, vault RNA; hVR1, human vRNAs.
previously (14, 21). Equal volume amounts were analyzed for protein and RNA content. Protein samples were solubilized in SDS sample loading buffer, fractionated on 7.5% SDS-PAGE, and transferred to Hybond-C (Amersham Biosciences) by electroblotting. Western blots were performed using an anti-rat vRNA polyclonal antibody (N2), affinity purified anti-TEP1 polyclonal antibody, and affinity purified anti-VPARP polyclonal antibody as described previously (21). Reactive bands were detected using horseradish peroxidase-conjugated secondary antibody and visualized by ECL (Amersham Biosciences). RNAs were purified by phenol/chloroform extraction and ethanol precipitation. Subsequently, the RNAs were fractionated on 8 M urea, 10% polyacrylamide gels and electroblotted to Zeta GT membrane (Bio-Rad).

The membrane was hybridized with a randomly primed human vRNA gene probe (specific activity 1 x 10^6 cpm/μg) according to the manufacturer’s recommendation. Hybridized bands were visualized by autoradiography.

UV-cross-linking assays were performed following the protocol of Dreyfuss and colleagues (22). Total rat kidney epithelial cell extracts were prepared from 10^7 cells by sonication with a microtip sonicator (Fisher 550 Sonic Dismembrator) for 2-5 s at setting 2. Debris was pelleted at 15,000 × g for 15 min, and the cleared supernatant was further analyzed. Sonicate extracts (50–75 μg) in a 50-μl final volume were exposed to a 15-watt germicidal lamp (Sylvania G15T8 bulb) at a distance of 4.5 cm for 15–20 min at 4 °C. To determine whether the same proteins could be cross-linked to the vRNA in vivo, live rat kidney epithelial cells (confluent 100-mm dishes) were irradiated for 3 min and then harvested and sonicated as above. Cross-linked RNA-protein complexes were solubilized in SDS sample loading buffer and fractionated on 7.5% SDS-PAGE. The fractionated RNA-protein complexes were electroblotted to a Hybond-C nitrocellulose membrane, which binds protein and cross-linked RNA-protein complexes but not to RNA alone. The membrane was hybridized with a randomly primed rat vRNA gene probe overnight at 65 °C according to the manufacturer’s recommendation. The nitrocellulose filter was washed twice with 1× SSC, 0.1% SDS at room temperature for 15 min. Hybridized bands were visualized by autoradiography.

Chromatography—Twelve milligrams of S100 extract from rat kidney epithelial cells was fractionated on a 2-ml DEAE-Sepharose column (Fast Flow, Amersham Biosciences) previously equilibrated in buffer A (50 mM Tris-Cl (pH 7.4), 1.5 mM MgCl2, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10% glycerol) containing 75 mM NaCl. The column was eluted with 16 ml of 75–500 mM NaCl linear gradient in buffer A by gravity, and 0.5-ml fractions were collected. Protein containing fractions were identified by Bradford assays and analyzed by UV-cross-linking (50 μl) as described above. Western analysis (15 μl), and Northern analysis of RNA extracted from fractions (50 μl). Western blots were probed with the human prototype anti-VEP1 serum Ze to detect the La autoantigen (23). Secondary antibody was goat anti-human antibody conjugated with horseradish peroxidase (Roche Molecular Biochemicals).

Label Transfer—The rat vRNA gene was transcribed with T3 RNA polymerase from a linearized plasmid DNA template. DEAE-Sepharose purified extract (25 μl) was incubated on ice for 20 min with 7.5 ng of 32P-labeled rat vRNA. The RNA-protein complexes were cross-linked with UV light as described above for 15 min followed by digestion with RNase A (40 μg/ml) and RNase T1 (1 unit/μl) at 37 °C for 15 min. Digested samples were solubilized in SDS sample loading buffer and fractionated on 7.5% SDS-PAGE. Dried gels were exposed to film to detect the La autoantigen (23). Secondary antibody was goat anti-human antibody conjugated with horseradish peroxidase (Roche Molecular Biochemicals).

Immunoprecipitation—A one-step immunoprecipitation from rat kidney epithelial cell sonic extracts was carried out. The precleared protein extract (230 μl) was incubated for 1 h at 4 °C with protein A-Sepharose beads and 10 μl of either human antiserum, Ze (anti-La) or Sto (anti-Ro). These human sera also recognize rat epitopes of La and the 60-kDa Ro/SS-A proteins, respectively. Immunoprecipitations were carried out in 250 mM Tris-Cl (pH 7.4), 5 mM EDTA, pH 8.0, 150 mM NaCl, 0.5% Nonidet P-40, 0.1% SDS, 0.5% deoxycholic acid, 0.02% sodium azide. The beads were washed five times with NET2 + F buffer. The unbound and first bead wash was saved for further analysis. The RNA from precipitates unbound and first wash was extracted and analyzed by Northern blot analysis (see above).

Gel Shift Analysis—His-T7-tagged La (human) was expressed in Echerichia coli using the pET system (Novagen) and purified on a His-bind affinity column. The human vRNA gene was transcribed with RNA T7 polymerase using a DNA template designed to maintain the native 3'-UUUU-OH terminus of the human vRNA (hVR1). La/vRNA binding reactions were incubated for 30 min at room temperature in a total volume of 10 μl and contained 10 fmol of 32P-labeled hVR1 in binding buffer (25 mM Tris-Cl (pH 7.8), 100 mM NaCl, 3 mM MgCl2, 0.5 mM dithiothreitol, 0.1 mM EDTA, 0.5% Nonidet P-40). Reactions containing cold competitor RNA were incubated for 5 min at room temperature prior to the addition of labeled hVR1. Samples were loaded onto a 4% non-denaturing gel (37.5:1 acrylamide:bisacrylamide) and electrophoresed for 45 min at 200 volts. Dried gels were exposed to PhosphorImager screens (Amersham Biosciences) to visualize labeled RNA and shifted complexes.

RESULTS

TEP1, VPARP, and vRNA Are Not Exclusively Complexed with the Vault Particle—Subcellular fractionation was used to determine the distribution of the vault components (MVP, VPARP, TEP1, and vRNA). HeLa cells were fractionated into nuclear (N), S100 (S), and P100 (P) fractions (Fig. 1A) (14, 21). Western blot analysis was carried out with polyclonal anti-MVP, anti-TEP1, and anti-VPARP affinity-purified antibodies. This analysis revealed that 85% of MVP is present as the macromolecular complex (the vault particle), which pellets with the microsomal fraction, and 5% is found in the nuclear fraction (Fig. 1A, MVP panel, compare lanes N and P). Both TEP1 and VPARP are found in all three fractions, although the level of TEP1 in the nuclear fraction is low in this analysis (Fig. 1A). Northern blot analysis of RNA extracted from these fractions revealed that most of the human vRNAs (hVR1–3) are not associated with the vault complex but instead are present in the S100 fraction (Fig. 1B). This finding prompted us to determine whether the vRNA might be interacting with other RNA-binding proteins in the S100 fraction.

The vRNA Is Found in a Small RNP Complex—To determine whether the vRNA complexes with other proteins, we utilized a UV-cross-linking hybridization assay (22). In this assay, total cell sonic extracts or live cells were treated with UV light covalently bonding the vRNA to any associated protein (see “Experimental Procedures”). RNA-protein complexes were fractionated on SDS-PAGE and blotted to a nitrocellulose membrane. Only a small portion of the vRNA becomes cross-linked to protein because of the low efficiency of UV-cross-linking, and these RNA-protein complexes bind to the nitrocellulose membrane. The membrane was hybridized to vRNA probe, and hybridized bands were visualized by autoradiography. As shown in Fig. 2, when total cell extracts and live cells are similarly analyzed, the vRNA cross-links to protein forming two vRNA-protein complexes (RNP I and RNP II), and these complexes are only detected after treatment with UV light. Given its approximate size (Mr, >250), RNP II was presumed to be a vRNA-TEP1 complex and was not further analyzed. The
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identification of a $M_r \sim 80$ vRNA-protein complex (RNP I) was intriguing and suggested that the vRNA may have an additional role outside of the vault particle. We have partially purified this vRNA-protein complex by fractionation of an S100 extract on a DEAE-Sepharose column. Fractions were initially analyzed using the UV-cross-linking/hybridization assay (Fig. 3A). This complex was further analyzed by incubating the DEAE-Sepharose purified extract with in vitro transcribed [P-32]-labeled rat vRNA, and the vRNA-protein complexes were treated with UV followed by digestion with a RNase mixture (see “Experimental Procedures”). By label transfer, we determined that the vRNA interacts with a protein of $M_r \sim 50$ (Fig. 4). Given that many unprocessed RNA polymerase III-transcribed RNAs are known to associate with the 47-kDa La/SS-B protein (24), it seemed reasonable to test this possibility. Using the anti-La serum Ze, we analyzed the DEAE-Sepharose column fractions by Western blot analysis and found that La immunoreactivity coincided with the same fractions that contained the RNP I and RNP II complex (Fig. 3B). This and the label transfer data suggested that the vRNA-associated protein in this complex is La.

vRNA Interacts Both in Vivo and in Vitro with the La Autoantigen—To further analyze the association of the vRNA with La, rat kidney cell sonic extracts were immunoprecipitated with anti-La serum Ze or anti-Ro serum Sto (both are human autoimmune sera). Sto recognizes the cytoplasmic RNP containing the 60-kDa Ro protein and the Y1-Y5 small RNAs (24–26). The RNA was extracted from immunoprecipitates and analyzed for the presence of the vault vRNA (Fig. 3C). Thus, we were able to show that La co-fractionates with vRNA and the $\sim 80$-kDa vRNA-protein RNP I complex (Fig. 3, A–C). This and the label transfer data suggested that the vRNA-associated protein in this complex is La.

with increasing amounts of His-T7-tagged La protein (Fig. 5B, lanes 2–4). A labeled band migrating slower than the vRNA was observed in the presence of La (Fig. 5B, solid dot). The intensity of this shifted band increased with increasing La concentration. The specificity of complex formation was analyzed in the presence of various competitors including cold vRNA, poly(U), and poly(A) (Fig. 5B, lanes 5–7, respectively). As expected, both cold vRNA and poly(U) were able to compete for binding of the His-T7-tagged La protein, whereas poly(A) was not. Using the anti-T7 monoclonal antibody to the epitope-tagged La protein, the vRNA-La complex could be supershifted (Fig. 5B, lane 8, asterisk). These data demonstrate a direct interaction of vRNA with the La RNA-binding protein in vivo and in vitro.

La Co-purifies with Rat Liver Vaults—Vaults purified from rat liver cell sonic extracts were immunoprecipitated with anti-La serum (Ze) and analyzed in Western blot analysis of purified rat liver vaults (Fig. 6B).
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La interacts in vivo and in vitro with the vRNA. A, rat cell sonic extracts were immunoprecipitated with either Ze, an anti-La (lanes 1–3) serum or with Sto, an anti-Ro (lanes 4–6) serum. Northern analysis of RNA extracted from immunoprecipitates (lanes 3 and 6), unbound lysate (lanes 1 and 4), and the first bead wash (lanes 2 and 5) was hybridized to detect the rat vRNA (vVR). B, mobility shift of hVR1-La complexes. Binding reactions contained 10 fmol of 32P-labeled hVR1 and 0, 4, 10, and 25 ng of purified La (lanes 1–4). Shifted complexes are indicated with a *. Competition reactions contained 25 ng of La protein and a 10-fold molar excess of the indicated cold competitor RNAs (lanes 5–7). 1 µg of a T7 monoclonal antibody (mAb) was used to supershift the La hVR1 complex (lane 8, asterisk).

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La co-purifies with vaults. A, vaults purified from rat liver were analyzed by Coomassie Blue staining. Arrows indicated the positions of the rat vault protein components. The ~180-kDa protein most probably is clathrin heavy chain as some coated vesicles co-purify with vaults. B, Western analysis of purified rat liver vaults with Ze anti-La serum. The La/SS-B protein is indicated with an arrow.

This result suggests that La associates with vaults in addition to interacting with the vRNA.

DISCUSSION

We have undertaken an analysis of the vRNA in extracts from tissue culture cell lines with the aim of examining its cellular role. Early characterization of vault particles revealed that vaults pelleted with the microsomal fraction at 100,000 × g (12). In this study, we refer to the microsomal fraction (100,000 × g pellet) as the P100 fraction, and we refer to the supernatant as the S100 or non-vault cytoplasmic fraction. As a first step in this analysis, we show that although most of the major vault protein is associated with the P100 fraction, TEP1, VPARP, and the vRNA are associated as well with the S100 fraction and in varying amounts with the nuclear fraction. This finding suggested that these components may have additional roles outside of the vault particle and that the vRNA may associate with other proteins. We have used a UV-cross-linking/hybridization assay to identify proteins that interact with the vRNA. We have partially purified a 50-kDa protein that cross-links to the vRNA in the S100 fraction and identified it as the La protein. Anti-La antibodies are found in the sera of patients with systemic lupus erythematosus and Sjogren’s syndrome (24). La is a ~47-kDa phosphoprotein that associates with small untranslated RNAs during their processing and incorporation into larger RNP complexes (15, 16). The association of La with these small RNAs is thought to occur largely through La binding to polyuridine-rich sequences, usually found at the 3′-end of unprocessed RNA polymerase III-transcribed RNAs. There are many precursor RNAs that have been identified in La RP complexes including 7 S RNA, tRNA, 5 S RNA, U6 RNA, Y RNAs, and the virally encoded RNAs, VA (1 and II) and EBER (1 and 2) RNAs (reviewed in Ref. 20). La association with these transcripts is thought to be transient, because most of the 3′-uridine-rich sequences are lost during maturation of the transcripts. La binding to the vRNA may be more stable because the vRNA transcript does not appear to undergo further processing after synthesis, and thus the 3′ end has an intact uridylicate tail (13). Indeed La interaction with vRNA in vitro can be competed with poly(U) but not poly(A), suggesting that binding to vRNA is through the 3′-UUU-UH tract. Two results indicate a stable association of the La protein with vRNA to form a small RNP complex: one is that the anti-La serum co-immunoprecipitates vRNA and another is that La co-purifies with the vRNA in the S100 fraction through DEAE-Sephrose chromatography and glyceral gradient purification at 200 mM NaCl. TEP1 is also found in the non-vault-cytoplasmic fraction, and at least a portion of it probably interacts with vRNA to form the RNP II complex identified here. Previously, we have shown that TEP1 interacts with vRNA and telomerase RNA in a yeast three-hybrid assay. Unlike with La, the sequences/secondary structures of the vRNA required for its binding to TEP1 have not yet been determined. Interestingly, in addition to an RNA binding domain, TEP1 also contains a Walker A motif, which in La has been shown to interact with the 5′-ppp of pre-tRNAs (27). Because the vRNA is not capped and has a 5′-pppG, this motif may be another binding site for interaction with La and/or TEP1.

The co-purification of La with vaults suggests that La may be involved in the assembly of vRNA into vault particles. Potentially, a La-vRNA complex may be targeted to vaults whereupon the vRNA is then transferred to vault-associated TEP1. Another possibility is that a La-vRNA complex may interact with non-vault-associated TEP1 and that this larger complex would then be targeted to vaults. This latter model seems less probable because preliminary analysis indicates that La remains associated with vaults purified from the livers of Tep1-deficient mice (data not shown). Thus, the interaction of La with vaults may not be dependent on TEP1. Previously, we have shown that in the absence of Tep1, the vRNA does not stably associate with vaults and that the stability of vRNA is greatly reduced in Tep1-deficient mouse embryonic fibroblasts (10). Although a La-vRNA complex may be targeted to vaults in both wild type and Tep1-deficient mice, vaults lacking TEP1 may be unable to then bind to the vRNA, even if it is brought within close proximity.

The precise subcellular localization of La is controversial. Most immunolocalization studies place La in the nucleus (reviewed in Ref. 20). However, La has also been identified in both the cytoplasm and in the nucleolus (20). In contrast, vaults
have a predominately cytoplasmic distribution both biochemically and by immunofluorescence. Our cell fractionation method used in this study would result in significant contamination of the S100 fraction with nuclear components; thus, the presence of the vRNA and La in this fraction cannot be used as evidence of a cytoplasmic distribution. It is possible that a portion of the vRNA is “stored” in the nucleus or perinuclear region or that all of the vRNA in this fraction could represent newly synthesized RNA. Recently, we have found that a portion of each of the individual vault components localizes to the nucleolus. Thus, it is not yet clear where a La-vRNA complex might assemble into vaults. The present findings indicate that only a fraction of cellular vRNA is found in vaults and that another fraction exists in at least two smaller RNP complexes: one containing La and the other presumably containing TEP1. These data suggest that there may be a dynamic relationship between the vRNA and vault particle and that La could mediate this interaction by acting as a chaperone.

Acknowledgments—We thank Sujna Raval-Fernandes and Nil Emre for critical reading of the paper and helpful discussion.

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