Multiple Human Vault RNAs
EXPRESSION AND ASSOCIATION WITH THE VAULT COMPLEX

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Human vaults are intracellular ribonucleoprotein particles believed to be involved in multidrug resistance. The complex consists of a major vault protein (MVP), two minor vault proteins (VPARP and TEP1), and several small untranslated RNA molecules. Three human vault RNA genes (HVG1–3) have been described, and a fourth was found in a homology search (HVG4). In the literature only the association of hvg1 with vaults was shown in vivo. However, in a yeast three-hybrid screen the association of hvg1, hvg2, and hvg4 with TEP1 was demonstrated. In this study we investigated the expression and vault association of different vault RNAs in a variety of cell lines, including pairs of drug-sensitive and drug-resistant cells. HVG1–3 are expressed in all cell lines examined, however, none of the cell lines expressed HVG4. This probably is a consequence of the absence of essential external polymerase III promoter elements. The bulk of the vault RNA associated with vaults was hvg1. Interestingly, an increased amount of hvg3 was bound to vaults isolated from multidrug-resistant cell lines. Our findings suggest that vaults bind the RNA molecules with different affinities in different situations. The ratio in which the vault RNAs are associated with vaults might be of functional importance.

The vault complex, with a molecular mass of 13 MDa, is the largest intracellular ribonucleoprotein particle described to date. Fifteen years ago, vaults were first observed in preparations of clathrin-coated vesicles from rat liver as unusual ovoid particles that displayed highly regular dimensions possessing a complex barrel-shaped morphology. The structures were named “vaults,” a term that describes the morphology of the particles, which contain multiple arches reminiscent of vaulted ceilings in cathedrals. Since then, vaults of nearly identical size and morphology have been reported to occur in phylogenetic groups as diverse as mammals, avians, amphibians, slime molds, echinoderms, mollusks, and protozoa. The mammalian vault particle consists of multiple copies of a 100-kDa major vault protein (MVP1), the minor vault proteins of 193 and 240 kDa, and small untranslated RNA molecules of 88–141 bases. The precise cellular function(s) of the vault complex are not yet completely clear (for reviews see Refs. 1–3).

The bulk of the vaults appears to reside in the cytoplasm. However, there are reports that describe vaults to be associated with the nucleus (4–6). Several groups reported on the involvement of vaults with intracellular transport (7–9) and nucleocytoplasmic transport (4–6). In addition, other lines of evidence suggest that vaults may function in intracellular detoxification processes and as a consequence function in multidrug resistance (MDR). Expression of MVP reflects the chemoresistance profile of many tumor cell lines and untreated cancers (3, 10–12). Furthermore, it was shown in several MDR cell lines that not only MVP was up-regulated but other vault components as well (13, 14). The most compelling data that link vaults to MDR comes from Kitazono et al. (15, 16). They used MVP-specific ribozymes in SW620 cells, which were induced by sodium butyrate to overexpress MVP. In this setting it was demonstrated that the reduction of MVP expression reverses the drug-resistant phenotype of the sodium butyrate-treated cells. It is still unclear by which molecular mechanism vaults function in MDR. However, the experiments by Kitazono et al. suggest that vaults play a role in MDR by blocking or preventing the accumulation of the anthracycline doxorubicin in the nucleus.

The mammalian vault complex is assembled into a typical hollow barrel-like structure with an 8-2-2 symmetry. It has an invaginated waist and two protruding caps, which most likely consist of the minor vault proteins and the vault RNA (17–20). The major vault protein is believed to be the main structural determinant of the complex, comprising 70% of its molecular mass. Each vault particle is composed of 96 MVP molecules, 8 molecules of p193, 2 molecules of p240, and at least 6 molecules of vault RNA. The p193 subunit contains a distinct domain with similarity to the catalytic domain of poly(ADP-ribose) polymerase (PARP). It was subsequently demonstrated that p193 catalyzes the ADP-ribosylation of itself and MVP (21). No other substrates for p193 have been described so far. Because of the PARP activity of p193 it was renamed vault PARP (VPARP). The p240 subunit was found to be identical to a previously described component of the telomerase complex, the telomerase-associated protein 1 (TEP1) (22, 23). The precise role of this subunit within the telomerase complex is not yet clear except that it is able to bind telomerase RNA. In a three-hybrid system TEP1 was shown to associate with vault RNA (22). Recently it was found that TEP1 is required for a stable association of the vault RNA with the vault complex (20).

The role of the vault RNA is still an enigma. However, based on experiments in which degradation of the vault RNA by RNase treatment did not lead to morphological changes, the
vault RNA is more likely to be a functional rather than a structural component (17, 19). Three related human vault RNA genes (HVG1–3; GenBank® accession numbers: AF045143, AF045144, and AF045145) have been described; a fourth gene was found in a homology search (HVG4) (13, 22). It was estimated that about 20% of the expressed vault RNA is associated to the vault complex (13). In a three-hybrid analysis the association of hvg1, hvg2, and hvg4 to TEP1 was shown (22). However, the only vault RNA of which an association with vault RNAs. The probes were end-labeled with [γ-32P]dATP. Hybridizations were performed at 55 °C in Church buffer for 4–6 h. Subsequently, the membranes were washed twice at room temperature in 2 × SSC, 0.1% SDS (20 × SSC: 3 × sodium chloride, 0.3 M trisodium citrate dihydrate, pH 8) and once in 5 × SSC, 0.1% SDS for 5 min at 55 °C. Blots were exposed to Kodak X-Omat AR film (Kodak, Rochester, NY) at −80 °C or exposed to a PhosphorImager screen (Molecular Dynamics, Sunnyvale, CA) for quantification. Afterward the blots were stripped and reprobed with a 5 S RNA-specific oligonucleotide (5'-TTCTCCATCAAGTACTAAGACACAC-3') as a control for equal loading.

Cloning and Sequencing of Human Vault RNA Genes—Putative human vault RNA genes were cloned by PCR using two primers, based on conserved regions in the rat and bullfrog vault RNA sequences (31). The forward primer was 5'-AGCTCACTGTTGATGACACGCGAAG; and HVG4, 5'-AGCTCACTGTTGATGACACGCGAAG; and HVG3, 5'-AGCTCACTGTTGATGACACGCGAAG; and HVG2, 5'-AGCTCACTGTTGATGACACGCGAAG and HVG1, 5'-AGCTCACTGTTGATGACACGCGAAG. Alternatively, a universal probe (5'-GCCCAGG- GGTTCGCAAG) was used, encompassing a region shared by all human vault RNAs. The probes were end-labeled with [γ-32P]dATP. Hybridizations were performed at 55 °C in Church buffer for 3–4 h. Subsequently, the membranes were washed twice at room temperature in 2 × SSC, 0.1% SDS (20 × SSC: 3 × sodium chloride, 0.3 M trisodium citrate dihydrate, pH 8) and once in 5 × SSC, 0.1% SDS for 5 min at 55 °C. Blots were exposed to Kodak X-Omat AR film (Kodak, Rochester, NY) at −80 °C or exposed to a PhosphorImager screen (Molecular Dynamics, Sunnyvale, CA) for quantification. Afterward the blots were stripped and reprobed with a 5 S RNA-specific oligonucleotide (5'-TTCTCCATCAAGTACTAAGACACAC-3') as a control for equal loading.

EXPERIMENTAL PROCEDURES

Cell Lines and Culture Conditions—The following human cell lines were used: the non-small cell lung carcinoma cell line SW1573, its doxorubicin (DOX)-selected MDR variant SW1573/DOX120 (24); the small cell lung carcinoma cell line G34C4, its DOX-selected derivative GLC4/ADR (25); the multiple myeloma cell line 8226 S and its DOX-selected variant 8226 D40 (26); the epithelial cell line KB 3-1 and two colchicine-selected derivatives KB 8 and KB 8-5 (27); the colorectal adenocarcinoma cell line SW620, breast adenocarcinoma cell line MCF7 (28); cervix epithelioid carcinoma cell line HeLa, embryonal kidney cell line 293 transformed with adenovirus 5 DNA, and the bone marrow stromal cell line L885 (29). All cell lines (except for MCF7 and L885) were maintained in Dulbecco’s modified Eagle’s medium (Life technologies Inc., Paisley, Scotland) supplemented with 10% fetal calf serum, 1 mM pyruvate, and 50 μg/ml gentamicin at 37 °C under an atmosphere containing 5% CO2. The cells MCF7 and L885 were maintained in RPMI (Life technologies Inc.) supplemented with 10% fetal calf serum and 50 μg/ml gentamicin and cultured under the same conditions. Doxorubicin was added to SW1573/DOX120, GLC4/ADR, and 8226 S at concentrations of 120, 240, and 375 nM, respectively. Colchicine was added to KB 8 and KB 8-5 at concentrations of 12.5 and 25 μM, respectively.

Antibodies—Immunoblotting experiments were performed with the rabbit polyclonal anti-MVP and the mouse monoclonal anti-p193 (mAb 8226 S at concentrations of 120, 240, and 375 nM, respectively. Colchicine was added to KB 8 and KB 8-5 at concentrations of 12.5 and 25 μM, respectively.

RESULTS

Cloning and Analysis of Human Vault RNA Genes—To screen the human genome for genes encoding putative vault RNAs, a PCR was performed using primers based on the conserved flanking parts of the rat and bullfrog vault RNA. The amplified fragments of ~100 bp were cloned and analyzed by sequencing. The analysis of 27 independent clones revealed the four previously described vault RNA genes (HVG1–4). No additional genes encoding vault RNA genes were found. A BLAST search showed that HVG1–3 are arranged in a triple repeat structure on chromosome 5q33.1 (BAC clone 119j3 (LBNL H175)) and
HVG4 is localized on chromosome Xp11.2 (PAC 339A18). The sequences spacing the HVGs on chromosome 5, about 7600 bp between HVG1 and HVG2 and 7200 bp between HVG2 and HVG3, do not encode any open reading frames. The alignment of the four detected hvg species (Fig. 1A) clearly shows two conserved stretches at both ends, which contain polymerase III promoter elements (box A and box B boxes). In between these conserved regions there is a part of variable length with no significant homologous stretches. Notably, the HVG1 gene contains a small duplication of 47 bp after the stop codon (TTTT). This repeat includes a second B box and ends with a genuine TATA box at position −70, and two distal sequence elements at positions −340 and −440 (Fig. 1B). The upstream sequence of HVG4 is mutated to TCTT, it seems to be functional, because an HVG3 expression product of the right size (88 bases) could be detected.

Comparison of upstream sequences of HVG1–4 revealed a high degree of similarity between HVG1, 2, and 3. Several external polymerase III promoter elements could be identified: a TATA box at position −20, a proximal sequence element at position −70, and two distal sequence elements at −340 and −440 (Fig. 1B). The upstream sequence of HVG4 was totally different and did not contain any recognizable external promoter elements. The TATA box of HVG3 is different from the other HVGs, TACAAT instead of TATAAT.

Characterization of Human Cell Lines for MVP and VPARP Expression—The major vault protein is expressed in all cell lines examined, although expression levels vary considerably (Fig. 2). Low levels were found in the breast adenocarcinoma cell line MCF7 and the embryonal kidney cell line 293. MVP could only be detected in these cell lines if vaults were concentrated by immunoprecipitation (data not shown). MVP is overexpressed in a number of drug-resistant cancer cell lines such as the SW1573/2R120 and GLC4/ADR in which the drug resistance is not mediated by P-glycoprotein (10, 13). In general, the expression levels of VPARP (p193) closely follow the MVP levels as would be expected, because they are both components of the same complex. However, this is not the case in the 8226 S and 8226 D40 cell lines.

Three of the Vault RNA Molecules Are Expressed in Cell Lines—Northern analysis was used to determine the expression levels of the four vault RNA species. Individual vault RNAs were detected by oligonucleotide probes (see Fig. 1A) that hybridize exclusively to specific vault RNAs. A universal probe, corresponding to the reverse primer, hybridizes to the conserved box B element that is present in all vault RNAs (Fig. 3A). Identical Northern blots were hybridized with these probes and with a 5 S rRNA probe as a control for equal loading. There is expression of hvg1, 2, and 3 in all cell lines examined (Fig. 3B). Longer exposure times revealed low levels of hvg2 and hvg3 in the GLC4 and GLC4/ADR cells and hvg3 in the 8226 S and 8226 D40 cells. Note that two hybridizing bands
were observed with the HVG3 probe. The RNAs migrate so close together in the gel system that they cannot differ more than a few bases in length. It is not clear whether these RNAs represent two true variants of hvg3 or whether they are the result of degradation during the procedure. hvg4 was not detected in any of the cell lines. Hybridization of a Northern blot with the universal probe verified the results and showed two bands in most lanes. The top band corresponded to hvg1 of 98 bases and the lower band to the co-migrating hvg2 and 3, both 88 bases in length. Quantification of the signal in each lane and calculation of the ratio hvg1 versus hvg2/3 showed that the expression ratio is cell line-dependent, the ratio being more or less equal in HeLa and the SW1573 and 8226 S cells. A slightly increased ratio was detected in the respective resistant derivatives, indicating a higher expression level of hvg1. The KB series and the L88/5 cells contained approximately twice as much hvg1 as hvg2/3, whereas the SW620, MCF7, and in particular 293 expressed more hvg2/3 than hvg1.

Cytoplasmic Pool of Vault RNA Consists of hvg1–3—To check if the hvg species are present in a non-vault-associated fraction, we performed three consecutive immunoprecipitations with rabbit polyclonal anti-MVP bound to Protein A-Sepharose beads. On a Western blot equal portions of the precipitated proteins (1, 2, and 3) and the remaining supernatant (S) were loaded (Fig. 4A). No residual MVP signal could be detected in the supernatant, indicating that it was cleared from vault particles. From the same samples RNA was isolated that was converted to cDNA and used in a PCR with a primer set capable of amplifying all vault RNA species. Equal portions of the PCR product were loaded onto an agarose gel, which was stained with ethidium bromide (Fig. 4B). The signal of the amplified vault RNAs decreased with each immunoprecipitation, which is clearly visible in the case of the GLC4 and GLC4/ADR cells. However, in all cell lines there was a strong signal in the supernatant fraction (S), indicating the presence of a pool of vault RNA. Southern analysis on the S fractions revealed that in all cases the pool consists of hvg1, 2, and 3 (only S fraction of GLC4/ADR is shown).

All Expressed Vault RNA Species Associate with the Vault Complex—When all vault RNA species have the same affinity for the vault complex, one would expect the expression ratio of hvg1–3 to be similar to the ratio found associated with the vault complex. Therefore, we isolated RNA from immunoprecipitated vaults and determined the levels of the hvg species by
Northern analysis using the universal probe (Fig. 5). In several parental cell lines we found that the bulk of the associated vault RNA is hvg1. On average, 50% of the expressed vault RNA is hvg1, however, about 80% of the vault RNA found bound to the vault complex is hvg1 (Table I). Clearly the hvg expression ratio does not reflect the ratio in which hvgs are associated with the complex.

To further assess which vault RNA species are associated with the vault complex, we immunoprecipitated vaults from each cell line. Subsequently vault RNA was isolated and converted to cDNA. Vault RNA sequences were amplified by PCR, using the universal primers. Southern analysis using hvg-specific probes demonstrated that hvg1 is associated with the vault complex in all cell lines (Fig. 6A). Association of hvg2 and hvg3 with the complex was observed as well. The hvg3 signal was consistently increased in the multidrug-resistant cell lines when compared with the hvg3 level found in their drug-sensitive parents. This phenomenon was not seen on the hvg1 and hvg2 blots indicating that this effect cannot be attributed to the overexpression of vaults. Using this sensitive RT-PCR assay we did not detect an hvg4 signal, which is in agreement with the absence of hvg4 expression.

To confirm the semi-quantitative PCR results, we isolated RNA from immunoprecipitated vaults from a large number of GLC4 and GLC4/ADR cells (Fig. 6B). Again the bulk of the vault RNA associated with vaults was hvg1, as was clearly shown when the blot was hybridized with the universal probe. In this exposure no signal of hvg2 and hvg3 could be detected. When the Northern blot, after stripping, was incubated with the HVG3-specific probe, a clear hvg3 signal was observed after a long exposure time. The hvg3 signal was increased in the drug-resistant cell line.

DISCUSSION

Degradation of vault RNA does not give rise to morphological alterations of the vault complex (17, 19). Therefore, it is be-
lieved that vault RNA is of functional importance to the complex. Because there are indications of vaults playing a role in multidrug resistance (MDR), we reasoned that this might be (partially) mediated by the vault RNAs expressed and associated with the vault complex.

We screened the human genome for all putative vault RNA genes by PCR using oligonucleotides that overlap conserved internal polymerase III promoter elements. After sequence analysis of 27 independent clones, only the four previously described vault RNA species (HVG1–4) were found. We may conclude that in total there are four putative human vault RNA genes. hvg1 was already shown to be associated with the vault complex in vivo (13). hvg1, 2, 3, and 4 were shown able to associate with TEP1 in a yeast-based three-hybrid system (22). TEP1 is also capable of binding telomerase RNA. However, telomerase RNA cannot be bound to TEP1, which is incorporated in the vault complex, because we were unable to detect telomerase RNA by RT-PCR in immunoprecipitated vaults (data not shown). It is not entirely clear whether vault RNA interacts with other vault components, but it was demonstrated by UV cross-linking that vault RNA primarily interacts with the minor vault proteins and not with MVP (13). The questions we focused on in this study are: Which vault RNA species are expressed in human cells, and which are associated to the complex in vivo? And is there a relation between vault RNA association and the MDR phenotype of human cancer cell lines?

A GenBank search with the HVG sequences showed that the genes encoding HVG1–3 are arranged in a triple repeat on the long arm of chromosome 5 at position 5q33.1 whereas the HVG4 gene is located on the X chromosome at position Xp11.2. All four genes have the typical polymerase III internal type-2 A and B box elements, but only the genes for hvg1–3 harbor external type-3 TATA, proximal, and distal sequence elements. The hvg sequences diverge and are unique in the region between A and B box elements. The distance between these elements is 41 bases in HVG1, 31 bases in HVG2 and HVG3, and 44 bases in HVG4. These differences may have consequences for the transcription efficiency (32). Most likely the HVGs have originated through gene duplication. It is noteworthy that the bullfrog has two vault RNA genes whereas the genomes of rat and mice have only a single gene. Because vault RNA genes of only a few organisms are available, it cannot be concluded whether there was ancestrally a single gene or multiple copies. Interesting in this respect is the fact that a repeat of the last 46 base pairs of the HVG1 gene directly follows its stop sequence TTAT. If the first stop would be absent, this would result in a vault RNA containing one A box and two B boxes with a size very similar to the vault RNA found in rodents (31). This is not the case in the genes for hvg2 and 3.

We set out to determine the vault RNA expression levels in various human cell lines, including drug-resistant ones that overexpress vaults and their drug-sensitive parental cell lines. It was found that most cell lines express hvg1 as well as hvg2 and 3, although the latter two at a lower level. In contrast, no transcript of HVG4 was found. Vilalta et al. (33) showed that transcription of rat vault RNA is dependent on both internal and external promoter elements. This is in agreement with the fact that we did not observe expression of HVG4 in the 14 cell lines examined. Although the HVG4 gene resembles the other HVGs closely and contains the type-2 internal promoter elements, the external promoter elements are lacking. When vaults were depleted from a cell lysate by subsequent immunoprecipitations, a pool of free vault RNA remained in the vault-cleared lysate. This pool consists of all three vault RNA species.

When we investigated which hvg species were actually associated with the vault complex we found that all three expressed species were coimmunoprecipitated with intact vault particles. Clearly the bulk of the vault RNA bound to the vault complex was hvg1, but also hvg2 and hvg3 were detected. The expression level of hvg2/3 is comparable in both-sensitive and -resistant cells. However, a sensitive RT-PCR procedure combined with Southern analysis suggested that the amount of associated hvg3 was increased in vault complexes isolated from multidrug-resistant cell lines. These results were confirmed in a more direct experiment in which levels of the various associated hvg species were determined by Northern analysis. In agreement with previous experiments, higher levels of hvg3 were associated with vaults in MDR cell lines. These results indicate that the enhanced level of associated hvg3 may be mediated by a change in affinity of the vault complex for the vault RNA species in response to certain functional cues.

In a recent study Siva et al. (34) describe that the ovarian carcinoma cell line A2780, when transfected with MVP (35), not only showed elevated MVP levels but that VPARP and TEP1 were overexpressed as well. Because the hvgs are present in excess in a non-vascul-associated pool, they were supposed not to be rate-limiting for vault assembly. These vault-component-overexpressing cells did not appear to be more resistant to drugs than the parental cells. Consequently, Siva et al. concluded that vaults may be necessary for MDR but are insufficient and other mechanisms underlie vault-mediated MDR. Our results suggest that the ratio of the vault-associated hvg species may mediate the role of vaults in MDR.

In patients suffering from myelodysplastic syndrome and acute myeloid leukemia, a loss of chromosome 5 and partial chromosome 5 deletions is associated with poor prognosis (36, 37). Notably, the human vault RNA genes are located in the region that is frequently involved in these chromosomal deletions (5q31-5q34). Breakpoints in this region might disregulate vault RNA expression and change vault RNA ratios, which may influence vault function in MDR and as such influence treatment outcome in these patients. Further studies are necessary to test this hypothesis.

The data presented in this study demonstrate that of the four putative HVGs described in the literature only three are transcribed (HVG1–3). Furthermore, we show that only a part of the expressed hvg species is associated with the vault complex at a ratio that does not reflect the expression ratio. The bulk of the vault RNA attached to vaults is hvg1 and a small amount is hvg2 and 3. The increased level of vault-attached hvg3 in MDR cell lines implies that the ratio in which hvgs species are associated with the complex may determine its function.

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