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Characterization of MVP and VPARP assembly into vault ribonucleoprotein complexes☆

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Abstract

Vaults are barrel-shaped cytoplasmic ribonucleoprotein particles composed of three proteins: the major vault protein (MVP), the vault poly(ADP-ribose)polymerase (VPARP), and the telomerase-associated protein 1, together with one or more small untranslated RNAs. To date, little is known about the process of vault assembly or about the stability of vault components. In this study, we analyzed the biosynthesis of MVP and VPARP, and their half-lives within the vault particle in human ACHN renal carcinoma cells. Using an immunoprecipitation assay, we found that it took more than 4 h for newly synthesized MVPs to be incorporated into vault particles but that biosynthesized VPARPs were completely incorporated into vaults within 1.5 h. Once incorporated into the vault complex, both MVP and VPARP were very stable. Expression of human MVP alone in *Escherichia coli* resulted in the formation of particles that had a distinct vault morphology. The C-terminal region of VPARP that lacks poly(ADP-ribose)polymerase activity co-sedimented with MVP particles. This suggests that the activity of VPARP is not essential for interaction with MVP-self-assembled vault-like particles. In conclusion, our findings provide an insight into potential mechanisms of physiological vault assembly.

Keywords: Vault; MVP; VPARP; Protein stability; Protein–protein interaction; LRP

Vaults are predominantly cytoplasmic ribonucleoprotein particles originally identified in preparations of coated vesicles [1]. They have been conserved throughout evolution and have been found in numerous eukaryotic species [2].

The vault complex has a barrel-like structure with an invaginated waist and two protruding caps. The molecular mass of the vault is 12.9 ± 1 MDa and cryo-EM single-particle reconstruction has provided overall dimensions of 42×75 nm [3]. Mammalian vaults comprise three proteins, 96 molecules of the 100 kDa major vault protein (MVP), 8 molecules of the 193 kDa vault poly(ADP-ribose)polymerase (VPARP), and 2 molecules of the 240 kDa telomerase-associated protein 1 (TEP1) and at least 6 molecules of small untranslated

☆ *Abbreviations:* MVP, major vault protein; VPARP, vault poly (ADP-ribose)polymerase; VPARP-C, the C-terminal region of VPARP (amino acids 1504–1724); PARP, poly(ADP-ribose)polymerase; PC-MVP, protein C-tagged human MVP; GST-VPARP-C, GST fused VPARP C-terminal region; LRP, lung resistance-related protein; APMSF, (*p*-amidinophenyl)methanesulfonyl fluoride hydrochloride.

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RNAs of 88–141 bases [4–7]. Despite the characterization of its structural features, the cellular functions of the vault are still unknown [8–12].

Vault RNA (vRNA) is associated with the TEP1 protein and TEP1 is required for vRNA stability and its association with the vault particle [13]. Both vRNA and TEP1 are unlikely to be the structural components of the vault particle since degradation of vRNA by RNase treatment or knockout of TEP1 did not result in morphological alterations of the vault structure [4,6,13]. VPARP contains a domain similar to the catalytic domain of poly(ADP-ribose)polymerase (PARP) and catalyzes the ADP-ribosylation of itself and of MVP [7]. This is the only known enzymatic activity of the vault components. The C-terminal end of VPARP [amino acids (aa) 1562–1724] can bind to the N-terminal half of MVP [14]. MVP molecules interact with each other via their coiled-coil domains (aa 648–800) [14]. When rat MVP alone was expressed in insect cells that do not contain endogenous vault particles or vault proteins, the formation of particles with a distinct vault-like morphology that had characteristics similar to those of endogenous rat vaults was observed [15]. Human MVP was initially described as a 110 kDa protein that was overexpressed in P-glycoprotein-negative multidrug-resistant tumor cell lines and was initially named the lung resistance-related protein (LRP) [16,17]. Despite the fact that MVP levels are a major indicator of chemotherapeutic failure in various cancer cell lines [11,18], MVP-deficient mice do not show hypersensitivity to cytostatic agents [19,20].

Although the individual vault components have been identified and characterized, very little is known about the process by which this highly ordered, huge ribonucleoprotein structure is formed. To understand the mechanism of assembly of the vault complex, and the factors that mediate this process, we studied the fate of pulse-labeled vault proteins in human renal carcinoma ACHN cells that express high levels of vaults. We further examined the role of the PARP activity of VPARP in an in vitro interaction between self-assembled MVPs and the C-terminal MVP interaction domain of VPARP (aa 1504–1724) that lacks the PARP catalytic domain expressed in *Escherichia coli (E. coil)* cells. We demonstrate the kinetics of assembly of newly synthesized MVP into vaults; rapid incorporation of VPARP into vaults; stabilization of MVP and VPARP proteins within the vault complex; and the interaction of self-assembled MVP with a VPARP mutant lacking PARP catalytic activity.

Materials and methods

Materials. EXPRESS Protein Labeling Mix [^{35}S] was purchased from Perkin–Elmer Life Sciences. (Boston, MA). Protein G–Sepharose-4B was from Zymed Laboratories (South San Francisco, CA). Dulbecco's modified Eagle's medium (DMEM) without L-methionine was from Invitrogen (Carlsbad CA). BUTVAR B-98 was purchased from Electron Microscopy Science (Hatfield, PA).

Antibodies. A rabbit anti-MVP polyclonal antibody (polyAb) was prepared using a glutathione *S*-transferase (GST)-MVP (aa 694–794) fusion protein as an antigen [21]. LRP-56, a monoclonal antibody against MVP, was a kind gift from Dr. Rik J. Scheper (Academic Hospital, Vrije Universiteit, Amsterdam, the Netherlands) [17]. A rabbit antiserum against VPARP (aa 1471–1729) was a kind gift from Dr. Valerie A. Kickhoefer (University of California, Los Angeles, CA) and purified with an Affi-gel 10 column from Bio-Rad Laboratories (Richmond, CA) according to previously described methods [7]. The mouse monoclonal anti-GST antibody and species-specific anti-immunoglobulin antibodies conjugated to horseradish peroxidase were from Amersham Life Science (Buckinghamshire, UK).

Cell culture. The human renal carcinoma cell line ACHN, that expresses a high level of vaults, was cultured in M199 medium (Nissui Phamaceutical, Tokyo, Japan) containing 10% fetal calf serum (FCS), 2 mM L-glutamine, and 100 IU/ml penicillin at 37 °C under a humidified atmosphere containing 5% CO_2.

Construction of expression vectors. Human MVP cDNA was inserted into a pXB vector (Roche Molecular Biochemicals, Indianapolis, IN) to construct the protein C-tagged human MVP (PC-MVP) expression plasmid. The cDNA for the human VPARP-C terminal region (aa 1503–1294) was inserted into the pGEX-4T-1 vector (Amersham–Pharmacia Biotech, Piscataway, NJ) to express the GST fused VPARP C-terminal region (GST-VPARP-C).

Generation of MVP knockdown cell lines. MVP knockdown cell lines were constructed using an siRNA expression vector. The MVP targeting sequence was based on a 19-mer sequence (GGACA CAGCTAAGAGCCTC) in MVP cDNA (1320–1329 bp). 64-mer synthesized oligonucleotides for cloning into pSUPER.retro (Oligoengine, Seattle, WA) were annealed and ligated into the pSUPER.retro construct as described [22]. The vector was transfected into ACHN cells and the stably transfected clones, clone #5 (knockdown) and clone #14 (unchanged MVP expression), were selected with puromycin (Sigma, St. Louis, MO).

Fluorography and immunoblotting. Samples mixed with 5× SDS sample loading buffer [5% (W/V) SDS, 0.125 M Tris–HCl, pH 6.8, 25% (V/V) glycerol, and 25% (V/V) β-mercaptoethanol] were heated at 100 °C for 5 min and subjected to 7.5% SDS–PAGE. For fluorography, the gels were fixed, stained with Coomassie blue, and subjected to fluorography using ENLIGHTNING (Du Pont-NEN Research Products, Boston, MA). The dried gels were exposed to FUJI Medical X-Ray film (Fuji Photo Film, Kanagawa, Japan) at −80 °C.

For immunoblotting, transfer to PVDF membranes was performed, and the blots were incubated with antibodies following established procedures [21]. Reactive bands were detected using the enhanced chemiluminescence system ECL (Amersham–Pharmacia, Buckinghamshire, UK).

Subcellular fractionation and immunoprecipitation of denatured and intact vaults. ACHN cells were harvested and lysed with 1 ml buffer A (50 mM Tris–HCl, pH 7.4, 75 mM NaCl, and 1.5 mM $MgCl_2$) containing proteinase inhibitors [aprotinin and (*p*-amidinophenyl)methanesulfonyl fluoride hydrochloride (APMSF)] and 0.5% (V/V) NP-40 or 6 M urea. The lysate was vortexed, incubated on ice for 15 min, and centrifuged at 20,000*g* for 20 min. The supernatant was designated as the S20 fraction.

Vaults were denatured by treatment of the S20 fraction with 6 M urea followed by dialysis against buffer A containing 0.5% NP-40. Then 300 μl of S20 fractions from either urea-treated or non-treated samples were centrifuged at 100,000*g* for 1 h. The resulting supernatant was designated the S100 fraction and the pellet was designated the P100 fraction. S20 fractions (600 μl) were precleared with protein G–Sepharose 4B. Each precleared sample was divided into two aliquots. One was incubated with the LRP-56 antibody and the other with the

anti-MVP polyAb for 1 h. Protein G–Sepharose 4B was then added and the incubation was continued for a further 1 h. After washing 4 times with PBS containing 0.5% (V/V) Triton X-100 the immunoprecipitates were analyzed by immunoblotting with the anti-MVP polyAb as above.

Metabolic labeling and immunoprecipitation of VPARP, MVP, and vaults. The cells were plated in 35 mm dishes (1×10^6 cells per dish) and cultured for 24 h in M199 medium. Cells were washed once with PBS and methionine-free DMEM, and then starved in methionine-free DMEM containing 10% dialyzed FCS for 15 min. For pulse-chase experiments, after 10 min of labeling with 5550 kBq/ml [^{35}S]methionine in methionine-free DMEM, the monolayers were washed and refed with 2 ml M199 medium containing 10% FCS supplemented with 0.5 mM unlabeled methionine. The cells were harvested at the indicated times after labeling and then stored at −80 °C until use.

All subsequent steps were performed at 4 °C. The frozen cells were lysed in 300 μl buffer A containing 0.5% NP-40 as above. The S20 of labeled cells were precleared with protein G–Sepharose 4B for 1 h. To study the relationship between MVP and vaults, S20 was incubated with the LRP-56 antibody for 1 h, then protein G–Sepharose 4B was added and the incubation was continued for a further 1 h. After centrifugation to separate the supernatant and the beads, anti-MVP polyAb was incubated with the supernatant for 1 h, then protein G–Sepharose 4B was added and the incubation was continued for a further 1 h. All the beads were washed 4 times with PBS containing 0.5% Triton X-100. All of the samples were analyzed by fluorography.

To study the relationship between VPARP and vaults, the S20 fraction was incubated with protein G–Sepharose 4B-bound anti-VPARP antibody for 1 h. Washing and analysis were performed as described above.

To determine the half-life of vault proteins, ACHN cells were plated onto 100 mm dishes (1 or 0.5×10^5 cells per dish) and labeled with [^{35}S]methionine for 15 min as described above. After washing and re-feeding for 4 h, the cells were harvested at the indicated times, and immunoprecipitation with the LRP-56 antibody and fluorography were carried out as described above.

Negative staining and electron microscopy of vault-like particles from E. coli transformed with MVP cDNA. The preparation of vaults from ACHN cells, and vault-like particles from *E. coli* transfected with PC-MVP cDNA, was carried out according to a previously described method [3]. In brief, *E. coli* expressing MVP was sonicated in 20 volumes of Mes buffer (0.09 M Mes and 0.01 M sodium phosphate, pH 6.5) containing APMSF and aprotinin. After centrifugation at 20,000*g* for 20 min at 4 °C, the supernatant was loaded onto a discontinuous sucrose gradient [made of 5%, 10%, 20%, 30%, 40%, 45%, 50%, 55%, and 60% (W/V)] [2] and centrifuged at 80,000*g* for 1 h. The fraction from the 5% layer was then loaded onto a new discontinuous sucrose gradient (made of 10%, 20%, 30%, 40%, 45%, 50%, 55%, and 60%) and centrifuged at 80,000*g* for 16 h. The layers of 40%, 45%, and 50% were collected and diluted 1:4 with Mes buffer containing 1 mM dithiothreitol (DTT), APMSF, and aprotinin, and then centrifuged at 80,000*g* for at least 3 h to pellet vaults. The pellet was resuspended by homogenization in buffer A containing 0.5 g/ml CsCl, loaded on a CsCl step gradient (1.45, 1.50, and 1.70 g/ml) in SM buffer (100 mM NaCl, 8 mM $MgSO_4 \cdot 7\ H_20$, and 50 mM Tris–HCl, pH 7.5) and centrifuged at 55,000*g* for 24 h. The load fraction was divided into equal upper and lower portions, diluted 1:4 with buffer A, and centrifuged at 80,000*g* for 3 h. The pellet was resuspended in Mes buffer and analyzed by silver staining and immunoblotting. Preparation of samples for electron microscopy was carried out as described [15] with the modification of using a BUTVAR B-98 supporting films coated grid instead of the carbon coated grid. In brief, samples were absorbed onto the grids for 3 min, stained with 2% uranyl acetate for 4 min at 4 °C, and dried on a filter paper until viewed with a HITACHI H7000 electron microscope.

Interaction of GST-VPARP-C and PC-MVP. All steps were carried out at 4 °C. Lysates from *E. coli*-DH5α expressing GST-VPARP-C or PC-MVP were prepared by sonication in 30 ml Mes buffer (0.09 M Mes and 0.01 M sodium phosphate, pH 6.5) followed by centrifugation at 20,000*g* for 20 min to remove insoluble material. Equal volumes (0.6 ml) of lysates containing PC-MVP or GST-VPARP-C were mixed and incubated for 1 h. Lysates (0.5 ml) of PC-MVP, GST-VPARP-C, a mixture of the two lysates, or the S20 fraction from ACHN cells (used as a positive control) were loaded onto a discontinuous sucrose gradient [made of 5%, 10%, 20%, 30%, 40%, 45%, 50%, 55%, and 60% (W/V)] [2] and centrifuged at 80,000*g* for 1 h. The fraction from the 5% layer was then loaded onto a new discontinuous sucrose gradient (made of 10%, 20%, 30%, 40%, 50%, 55%, and 60%) and centrifuged at 80,000*g* for 16 h. The fractions were collected and analyzed by immunoblotting with the anti-GST antibody and the anti-MVP polyAb as described above.

Results

Characterization of MVP antibodies and urea disruption of vaults

Prior to the study of vault protein characteristics and vault assembly we first characterized the antibodies used in this study as to their ability to recognize vault proteins in intact or urea disrupted [15] vaults. As shown in Fig. 1A the polyclonal anti-MVP antibody immunoprecipitated the MVP protein of cellular lysates of ACHN cells both in the presence and absence of urea treatment. However, the previously described monoclonal anti-MVP antibody LRP-56 [23] could only recognize MVP when present in the intact vault particle.

To confirm that the MVP protein detected by the polyclonal antibody following urea treatment was derived from disrupted vaults we subjected the low speed supernatant of cell lysates (S20) to a high speed centrifugation. As expected, in the absence of urea treatment MVP was detected predominantly in the pellet (P100) fraction. Treatment of S20 cell extracts with 6 M urea followed by dialysis resulted in the transition of almost all of the MVP to the supernatant (S100) fraction (Fig. 1B). It suggests that it is necessary for all of vault's MVP to assemble before the LRP-56 antibody can recognize the appropriate structure. The differential recognition of MVP by the two anti-MVP antibodies therefore provides useful reagents with which to study the role of MVP in vault assembly.

Assembly of VPARP and MVP into the vault complex

To investigate the half-life of vault particles, ACHN cells were labeled with [^{35}S]methionine for 10 min, chased for 1–4 h, and harvested for lysis and immunoprecipitation of intact vaults with LRP-56. The efficiency of LRP-56 immunoprecipitations was confirmed to be quantitative by re-precipitation of the post-immunoprecipitation supernatants (data not shown). Analysis of the ^{35}S-labeled proteins in the LRP-56 immunoprecipitates by fluorography indicated that the 100 and

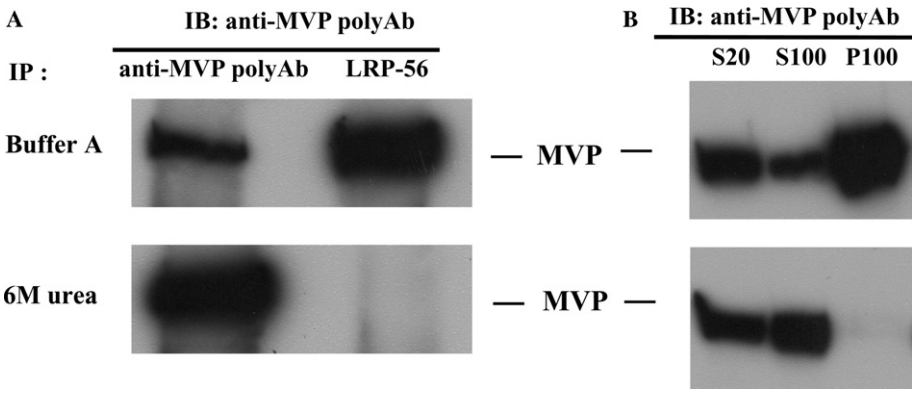

Fig. 1. Characterization of anti-MVP antibodies. (A) ACHN cell lysates (S20, 200 μl) were immunoprecipitated with the anti-MVP polyAb or the LRP-56 monoclonal antibody in the absence (Buffer A, top) or presence of 6 M urea treatment (bottom). The immunoprecipitates were electrophoresed and immunoblotted with the anti-MVP polyAb. (B) To confirm that urea treatment of cell lysates disrupted vault structure ACHN cell lysates (S20) were treated without, (Buffer A, top) or with, 6 M urea (bottom). Lysates (100 μl) were then centrifuged at 100,000*g* for 1 h and the pellet (P100) and 20 μl of the supernatant (S100) or of S20 were subjected to SDS–PAGE and immunoblotted with the anti-MVP polyAb.

200 kDa bands, but not the 240 kDa band, could be detected with this method (Fig. 2A). We confirmed that the 100 and 200 kDa bands represented the MVP and VPARP, respectively, by immunoblotting of the LRP-56 immunoprecipitates (data not shown). Labeled MVP was only weakly detected immediately following the 10-min labeling with [^{35}S]methionine and the intensity of MVP labeling increased in a time-dependent manner over a 4 h period. In contrast, substantial labeling of VPARP was already observed following 10 min labeling with [^{35}S]methionine and did not dramatically increase over the 4 h chase period.

Since the LRP-56 antibody can only immunoprecipitate intact vault particles, the lag time observed until the appearance of an intense MVP signal may indicate the time required for assembly of newly synthesized MVPs into vault particles. To investigate this hypothesis we examined the level of unincorporated MVP remaining in the supernatant following immunoprecipitation of the vaults with the LRP-56 antibody. Immunoprecipitation of the supernatant with the anti-MVP polyAb indicated that a considerable level of free [^{35}S]methionine-labeled MVP was detectable immediately following the 10 min pulse-labeling. The level of free MVP decreased in a time-dependent fashion concomitant with an increase in the level of labeled MVP detectable in LRP-56-precipitated intact vaults (Fig. 2B), suggesting that the free MVP is being incorporated into vaults.

The slow assembly of MVP into vault particles is in contrast to the apparent rapid incorporation of VPARP. To investigate the incorporation of VPARP into vaults lysates from ACHN cells, that were pulse-labeled and chased for 0–1.5 h, were immunoprecipitated with an anti-VPARP antibody. This antibody can recognize free VPARP and therefore can only detect non-vault-associated VPARP. Immunoprecipitation with this antibody

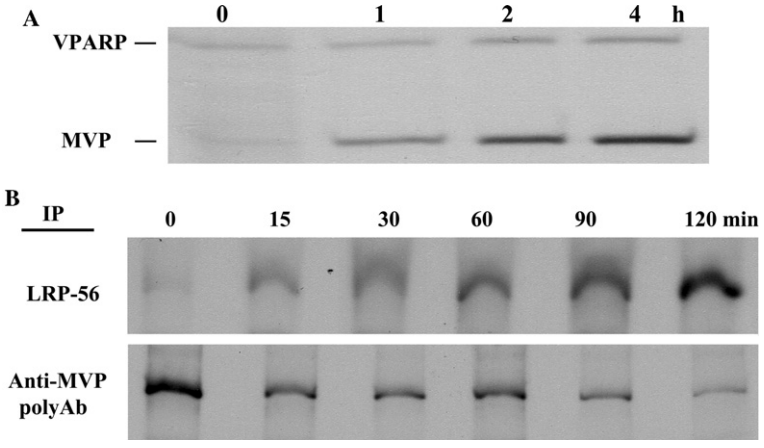

Fig. 2. Stability and incorporation of vault proteins. ACHN cells were metabolically labeled with 5550 kBq/ml [^{35}S]methionine for 10 min and chased for the indicated times. Cell lysates were immunoprecipitated with the LRP-56 antibody (A) or sequentially with the LRP-56 antibody (top) followed by immunoprecipitation of the LRP-56 depleted supernatant with the anti-MVP polyAb (bottom) (B). The precipitates were subjected to SDS–PAGE and fluorography. The migration positions of VPARP and MVP are indicated.

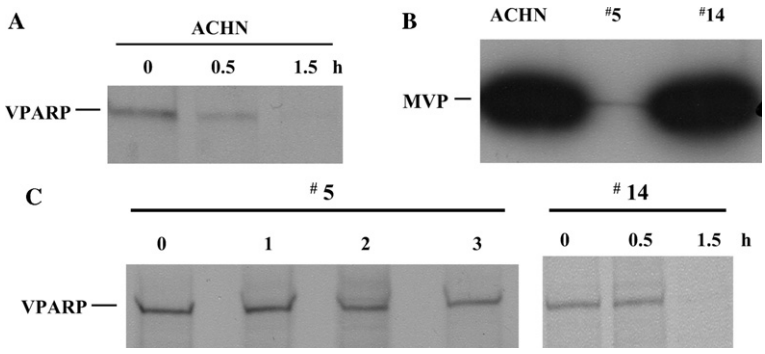

Fig. 3. Incorporation of VPARP into vaults. Parental ACHN cells (A), or an MVP-knock-down (clone #5) or control (clone #14) clone, established with MVP-targeting siRNA (C), were metabolically labeled with 5550 kBq/ml [^{35}S]methionine for 10 min and chased for the indicated periods. Cell lysates were immunoprecipitated with the anti-VPARP antibody and subjected to SDS–PAGE and fluorography. Expression of MVP in parental ACHN cells, and in the MVP-knockdown (clone #5) and control clone (clone #14), assessed by immunoblotting with the anti-MVP polyAb, is shown in (B). VPARP and MVP are indicated at left.

indicated that free, [^{35}S]methionine-labeled VPARP rapidly disappeared following labeling and was undetectable at 1.5 h (Fig. 3A). These data suggest either that free VPARP is very unstable or that most of the VPARP molecules are immediately incorporated into vaults. To ascertain which is the case, the stability of non-vault associated VPARP was studied in MVP-knockdown ACHN cells in which vaults cannot be assembled. MVP-knockdown ACHN cells were established using small interfering RNA (siRNA). Expression of MVP was dramatically down-regulated in the MVP-knockdown clone #5 compared to clone #14 or to the parental cell line (Fig. 3B). The level of VPARP in clone #5 and #14 was assayed following a 10 min pulse with [^{35}S]methionine and a chase for 0–3 h (clone #5) or 0–1.5 h (clone #14) by immunoprecipitation with anti-VPARP and fluorography. Newly synthesized VPARP disappeared after a 1.5 h chase in clone #14 as had been observed in the ACHN cells. However, labeled VPARP was still detectable in clone #5 even after a 3 h chase although the level of VPARP was slightly decreased (Fig. 3C). These data suggest that the rapid disappearance of free VPARP protein following labeling is due to its immediate assembly into the vault complex rather than to VPARP protein instability.

Stability of the MVP and VPARP proteins within vaults

We next examined the half-lives of MVP and VPARP within the vault particles. ACHN cells were labeled with [^{35}S]methionine for 15 min and chased for various times. Since most newly synthesized MVPs were completely assembled into vaults by 4 h, a time point of 4 h after pulse-labeling was arbitrarily assigned as time 0 for chase studies of the stability of MVP and VPARP within the vault complex. As shown in Fig. 4A, both the MVP and VPARP proteins were stable within the vault complex and no significant decrease of either protein was

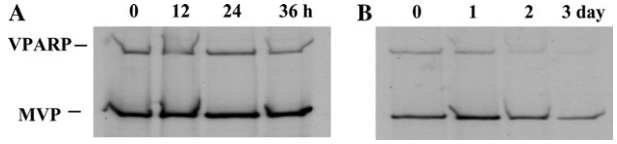

Fig. 4. Stability of vault proteins. ACHN cells, plated at a density of 1×10^6 (A) or 5×10^5 (B), were metabolically labeled with 5550 kBq/ml [^{35}S]methionine for 15 min and chased for the indicated times. The point 4 h after pulse labeling was arbitrarily assigned as time 0, since most of the labeled MVPs were assembled into vaults at this time point. Cell lysates were immunoprecipitated with the LRP-56 antibody and analyzed by SDS–PAGE and fluorography. VPARP and MVP are indicated at left.

observed, even after a 36 h chase. Extension of the chase over a 3 day period revealed that the apparent half-lives of MVP and VPARP within vault particles are at least 3 days and 40 h, respectively (Fig. 4B). As the average half-life of total ACHN cell protein is about 2 days (data not shown), MVP and VPARP are relatively stable once they are incorporated into vault particles.

Investigation of the role of PARP activity in incorporation of VPARP into vaults

We next studied whether PARP activity is involved in the incorporation of VPARP into vaults. For this purpose we used an in vitro assay of vault assembly that is based on the fact that the MVP protein alone, when expressed in *E. coli*, can form particles that are morphologically similar to vaults from ACHN cells when negatively stained and examined by electron microscopy (Fig. 5A). By a silver staining, MVP band alone was detected in *E. coli* vault-like particles, while two bands for MVP and VPARP were detected in vaults from ACHN cells (Fig. 5B). MVP bands were confirmed by anti-MVP polyAb (Fig. 5C).

A GST-tagged-C-terminal region of VPARP (aa 1294–1503, GST-VPARP-C) that includes a previously

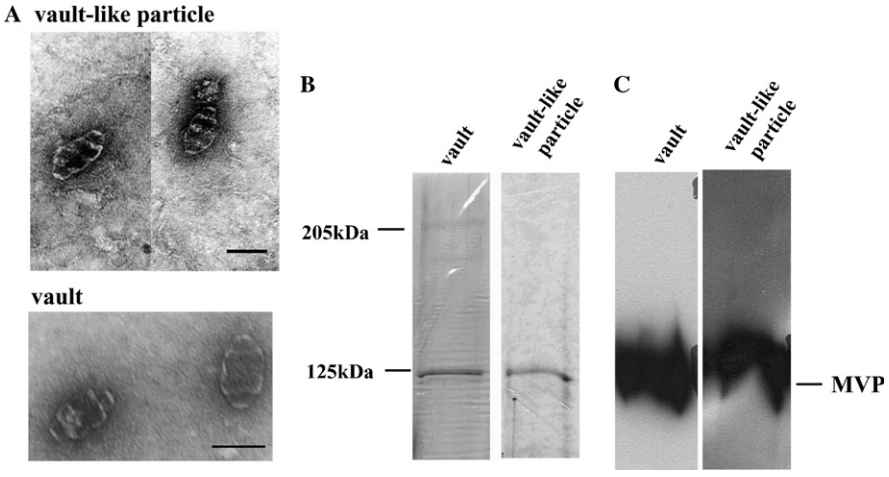

Fig. 5. Vault-like particles formed by MVP in *E. coli*. PC-tagged MVP was expressed in *E. coli* and purified. Self-assembly into vault-like particles and MVP expression was analyzed by negative staining and electron microscopy (A) and silver staining (B), and the proteins were confirmed by immunoblotting with the anti-MVP polyAb (C). Vaults purified from ACHN cells were also examined. The bar represents 50 nm.

identified MVP interaction domain but lacks the PARP domain [7,14] was also expressed in *E. coli*. Lysates of *E. coli* expressing either Protein C-tagged human MVP (PC-MVP) or GST-VPARP-C were mixed and incorporation of GST-VPARP-C into the self-assembled PC-MVP vault was assayed by sucrose step gradient centrifugation. As shown in Fig. 6, self-assembled PC-MVPs were distributed predominantly in the 40–50% sucrose layers in a similar pattern to vaults isolated from ACHN cells. GST-VPARP-C distributed mainly in the 5–20% layers. However, when GST-VPARP-C was incubated with PC-MVP prior to loading onto the sucrose step gradient, GST-VPARP-C was also found in the 45% and 50% sucrose layers. These data suggest that PARP activity is not essential for incorporation of GST-VPARP-C into the vault-like particles assembled by *E. coli*-expressed-PC-MVP.

Discussion

The high evolutionary conservation and wide species distribution of vaults suggest an important cellular function. However, as is the case with other large ribonucleoprotein particles, the physiological process by which vault complexes are formed, and the factors that mediate this process, is still unclear.

In this study, we first investigated the dynamic assembly of vaults using two antibodies that differentially interact with free, and vault incorporated, MVP. We could not determine whether the unincorporated MVP immunoprecipitated by the anti-MVP polyAb was a soluble or an aggregated form of MVP. We found that it took around 4 h for most of the pulse-labeled MVPs to be assembled into vaults in ACHN cells but that VPARP was rapidly incorporated into vaults. The con-

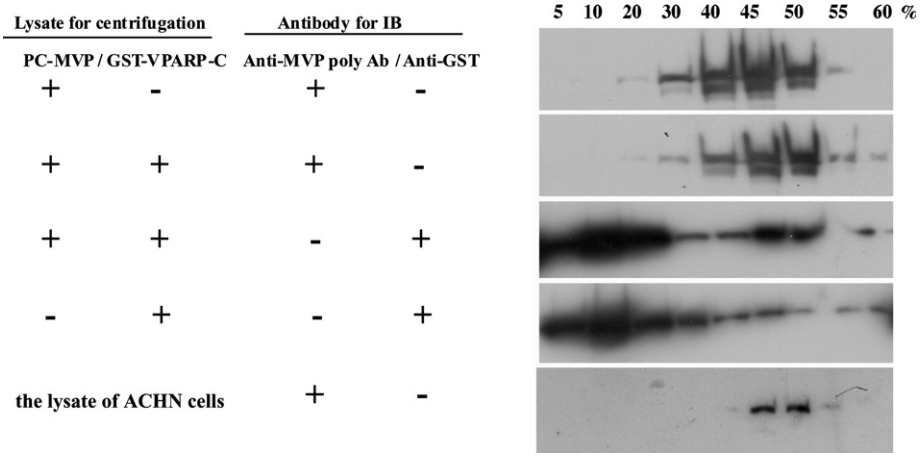

Fig. 6. Interaction of vault-like particles with the C-terminal region of VPARP in vitro. Lysates from *E. coli* expressing PC-MVP, GST-VPARP-C or a mixture of both, were centrifuged on a discontinuous sucrose gradient. One-twenty-fifth of each fraction was subjected to SDS–PAGE and immunoblotted with the anti-MVP polyAb or the anti-GST antibody as indicated. The lysate of ACHN cells as a positive control.

siderable period needed for MVP to be assembled into vaults suggests the existence of intermediates in the assembly process from newly synthesized MVP to incorporation into vaults. Further study is needed to elucidate the dynamic mechanism of MVP assembly and its regulation. Since calcium appears to be important for the folding and assembly of MVP molecules into complete vault particles [14], the intracellular concentration of calcium ions and the mechanisms by which calcium ion concentration is regulated might affect this process.

We found that the half-lives of MVP and VPARP in vault particles were longer than 3 days and 40 h in ACHN cells, respectively. The considerably long stability of MVP within the vault complex, together with the ability of *E. coli* expressed MVP to self-assemble into vault-like particles, supports the proposal that MVP is a structural protein [15].

The level of free, pulse-labeled VPARP decreased much faster than that of MVP in pulse chase studies (Figs. 2B and 3A). We have excluded the possibility that the free VPARP was rapidly degraded since free VPARP was detected even after a 3 h chase in MVP-knockdown cells whereas it had disappeared within 1.5 h in MVP expressing parental cells (Figs. 3A and C). These findings suggest that newly synthesized VPARPs are immediately incorporated into vaults without any delay.

Recently it was reported that there is a dynamic link between VPARP and vault particle [24]. By changing the temperature between 21 and 37 °C, reversible dissociation of VPARP from MVP molecules was able to be detected [24]. We could not find the decrease of the labeled vault-associated VPARP after at least 36 h. The stability of the VPARP in vaults implied relatively stable interaction between vaults and the vault-associated VPARP, and less interchange between vault-associated VPARP and the free VPARP at 37 °C.

It has been shown by yeast two-hybrid analysis that the N-terminal half of MVP binds to an MVP-interaction domain in the C-terminal end of VPARP [14]. This binding should not interfere with the self-association of MVP molecules which has been attributed to interaction via the coiled-coil domain located in the C-terminal half of MVP molecules [14]. These data also suggest that one MVP molecule can bind to one VPARP molecule. However, a vault complex consists of only 8 molecules of VPARP in contrast to 96 molecules of MVP [6]. Therefore, it is complete, or partial, MVP self-assembly that occur prior to VPARP binding to MVP thereby hiding VPARP binding sites. In this study, we have shown that newly synthesized VPARP can be incorporated into unlabeled pre-assembled vaults and that vault-like particles composed solely of MVP can interact with GST-VPARP-C. Our observations may therefore reflect the physiological process of vault assembly.

PARP activity is the only biochemical activity that has been detected in association with vault proteins, and both MVP and VPARP itself are substrates of the VPARP activity in vitro [7]. In this study, we found that PARP activity was not required for in vitro vault assembly. Although no PARP activity was detected in *E. coli* [25], VPARP, in which the PARP activity was deleted, could still interact with *E. coli*-expressed MVP self-assembled vault-like particles. This suggests that PARP activity may have an important role in the function rather than in the assembly of vaults although it cannot be ruled out that PARP activity might enhance assembly. This is consistent with the report that vault can be detected in VPARP knockout mice [26].

In conclusion, this study has defined a number of important parameters of vault assembly including the differential kinetics of incorporation of MVP and VPARP into vaults, the stability of MVP and VPARP proteins within the vault structure, and has also analyzed the requirement for PARP activity in vault assembly. These findings can be used as a basis for elucidation of the physiological mechanism of vault assembly and may help to clarify the cellular function of the vaults and thereby the potential role of MVP overexpression in multidrug resistance.

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