Assembly of Vault-like Particles in Insect Cells Expressing Only the Major Vault Protein*

Vol. 276, No. 26, Issue of June 29, pp. 23217–23220, 2001

Accelerated Publication

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 Vaults are the largest (13 megadalton) cytoplasmic ribonucleoprotein particles known to exist in eukaryotic cells. They have a unique barrel-shaped structure with bifold symmetry. Although the precise function of vaults is unknown, their wide distribution and highly conserved morphology in eukaryotes suggests that their function is essential and that their structure must be important for their function. The 100-kDa major vault protein (MVP) constitutes ~75% of the particle mass and is predicted to form the central barrel portion of the vault. To gain insight into the mechanisms for vault assembly, we have expressed rat MVP in the Sf9 insect cell line using a baculovirus vector. Our results show that the expression of the rat MVP alone can direct the formation of particles that have biochemical characteristics similar to endogenous rat vaults and display the distinct vault-like morphology when negatively stained and examined by electron microscopy. These particles are the first example of a single protein polymerizing into a non-spherically, non-cylindrically symmetrical structure. Understanding vault assembly will enable us to design agents that disrupt vault formation and hence aid in elucidating vault function in vivo.

MATERIALS AND METHODS

Plasmid Subcloning, Recombinant Baculovirus Preparation, and Protein Production—The His-T7 tag was amplified by polymerase chain reaction from the pET28a plasmid (Novagen) using the T7 primer (Life Technologies, Inc.) and TCAGCCATGGCATTGCTGCCAAG, which contains a NcoI site. The amplified His-T7 tag was subcloned into the NcoI site at the ATG start site of rat MVP (22). The His-T7-tagged rat MVP was subcloned into the pFASTBAC vector (Life Technologies, Inc.) at the XbaI and KpnI sites. The vsg-tagged rat MVP, described previously (23), was subcloned into the pFASTBAC vector using EcoRI. Recombinant baculovirus was then generated as directed in the Bac-to-Bac baculovirus expression system (Life Technologies, Inc.). Sf9 cells adapted for serum-free growth (Life Technologies, Inc.) were cultured at 28 °C on an orbital shaker rotating at 135–150 rpm. Cultures were seeded at 2 × 10⁶ cells/ml, inoculated with virus at a multiplicity of 3 and the pellets stored at −80 °C. Purification of Vault-like Particles from Sf9 Cells—Infected cells were resuspended in 10 volumes of buffer A (50 mM Tris-HCl, pH 7.4, 75 mM NaCl, 0.75 mM MgCl₂) containing 1% Triton, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride (PMSF), and a protease inhibitor mixture (2 μg/ml aprotinin, 0.5 μM benzamidine, 2 μg/ml chymostatin, vault poly(A)DP-ribose polymerase; TEP1, telomerase-associated protein 1; EM, electron microscopy; PMSF, phenylmethylsulfonyl fluoride; VLP, vault-like particle.

*This work was supported by National Institutes of Health Grant GM38097. 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 1734 solely to indicate this fact.

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¶ These abbreviations used are: MVP, major vault protein; VPARP, (VPARP) (4), and the telomerase-associated protein 1 (TEP1) (5) and one or more untranslated RNAs (6). Vaults have been implicated in the phenomenon of multidrug resistance and as prognostic markers for cancer chemotherapy failure (7, 8). One recent study has shown that the major vault protein is involved directly in the efflux of drugs from the nucleus (9). Although the majority of vaults are found in the cytoplasm, small amounts have been localized to the nuclear pore complexes (10). Recently a 31-Å resolution structure of the vault has been published indicating that vaults have a hollow interior consistent with a transport or sequestration function. Scanning transmission electron microscopic analysis has shown that 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 (11). Freeze-etch images of the vault on polylsine-coated mica show that each half of the vault midsection can open into eight distinct “petals” (3), which has lead to the proposal that vaults may open and close in vivo. The MVP is presumed to be present in 96 copies/vault, based on the observed symmetry of the particle and the estimate that MVP accounts for ~75% of the total protein mass in the particle. In many ways vaults are reminiscent of virus particles, in that they are MDa assemblies that have a protein shell composed of multiple copies of a single protein and have a large central cavity. The expression of specific viral proteins in baculovirus has become an invaluable tool for investigating the assembly of virus particles (12–14). There are numerous examples where expression of a single viral protein was capable of directing the formation of virus-like particles (12, 15, 16). However, other studies required expression of multiple viral proteins (13, 14). Currently nothing is known of the mechanisms by which vault particles are assembled within the cell. Toward this goal, we have expressed rat MVP in the Sf9 insect cell line using a baculovirus vector. Here we show that the expression of the rat MVP alone results in the formation of particles that have characteristics similar to endogenous rat vaults.

VLP, vault-like particle.
5 μM leupeptin, 5 μM pepstatin), incubated on ice for 20 min, and then homogenized 10 times with a type A Dounce. Unbroken cells, organelles, and membranes were pelleted by centrifugation at 20,000 × g (S20) for 15 min at 4 °C. Large protein complexes (including vaults) were collected by further centrifugation of the supernatant at 100,000 × g for 1 h at 4 °C. The pellet (P100) was resuspended by Dounce homogenization with 1 ml of buffer A containing 1 mM dithiothreitol, 1 mM PMSF, and the protease inhibitor mixture. This P100 fraction was then adjusted to 7% sucrose and Ficoll and centrifuged at 43,000 × g for 40 min at 4 °C. The supernatant was collected and diluted 1.3 with buffer A containing 1 mM dithiothreitol, 1 mM PMSF, and the protease inhibitor mixture and centrifuged at <100,000 × g for at least 3 h to pellet vaults. The pellet was resuspended by Dounce homogenization in 1 ml of buffer A containing 1 mM dithiothreitol, 1 mM PMSF, and the protease inhibitor mixture. To remove contaminating ribosomes, 500 μg of RNase A and 50 units of RNase T1 (Ambion) were added and incubated for 20 min at room temperature. The insoluble ribosomal proteins were then pelleted by centrifugation at 20,000 × g for 15 min at 4 °C. The supernatant was loaded onto a sucrose step gradient of 20, 30, 40, 45, 50, and 60% sucrose steps and centrifuged at 78,000 × g for 16 h. The fractions were collected and diluted 1:9 with buffer A containing 1 mM dithiothreitol, 1 mM PMSF, and the protease inhibitor mixture, and the vaults were pelleted by centrifugation at 100,000 × g for 3 h. The pellets were then resuspended in 200 μl of buffer A containing 1 mM dithiothreitol, 1 mM PMSF, and the protease inhibitor mixture and analyzed by silver staining and Western blot.

**Negative Staining and Electron Microscopy**—Sucrose gradient fractions were prepared for electron microscopy by absorption of samples onto fresh 1.8-nm-thick carbon film mounted on carbon-coated holey-film grids for 2–5 min at 4 °C. Following sample adsorption, grids were floated for 4 min on 1 ml of 1% uranyl acetate at 4 °C and dried on filter paper prior to viewing in a JEOL 1200EX microscope. The carbon-coated holey-film grids and carbon film were the gift of Dr. Sergey Ryazantsev, Department of Biological Chemistry, UCLA School of Medicine. Micrographs were produced digitally from EM negatives (3 × 4 inch) scanned at 1500 dpi resolution in TIFF format using a Scitex scanner and processed using Adobe Photoshop 6.0 software.

### RESULTS AND DISCUSSION

**Expression of Rat MVP and Formation of Vault-like Particles in Insect Cells**—To study vault assembly, the baculovirus system was used for the expression of epitope (either vsvg or His-T7)-tagged rat MVP in Sf9 cells. Cell extracts from uninfected (control) and infected Sf9 cells were fractionated into a soluble (S100) and particulate (pellet, P100) fraction by centrifugation at 100,000 × g. Epitope-tagged rat MVP expressed in infected Sf9 cells was found abundantly in the P100 (Fig. 1A) similar to mammalian cell expression (2, 7). This finding suggested that the expressed MVP was either aggregation or assembling into some type of particle in vivo. No endogenous MVP was detected in cell extracts, S100, or P100 from uninfected (control) Sf9 cells (Fig. 1A) using an anti-MVP antibody raised to a conserved region in MVP, amino acids 593–805 (17). Several attempts were made to isolate vaults from uninfected Sf9 cells using a modified version of the Dictyostelium (a cellular slime mold) vault purification protocol (see “Materials and Methods”). However, during the course of the purification, no proteins were detected that were cross-reacted with the anti-MVP antibody described above. In addition, analysis of the purified material by electron microscopy did not reveal any vault-like particles (data not shown). Finally, no MVP homologue has yet been detected in any insect genome. Taken together the above results suggest that either Sf9 cells do not contain vaults or vaults in this organism are so highly divergent as to no longer resemble chemically or physically the vault particle that has been highly conserved from slime mold to man (1).

When Sf9 cells expressing tagged MVP were subjected to the vault purification protocol, analysis of the purified material showed that the tagged rat MVP was found predominantly in the 40 and 45% sucrose layers (Fig. 1B) similar to what is found when vaults from numerous tissue culture cell lines are examined (7). In addition, the MVP in the 45% sucrose layer constituted ~95–99% of the material that was silver-stained (Fig. 1B, upper panel). This fractionation of the rat MVP in the 40 and 45% sucrose layers is consistent with the expressed protein forming a large particle. Expressed rat MVP was also found in the 50 and 60% sucrose fractions, similar to our earlier studies. Because of the abundant protein expression, one concern was that the spread of material from 40–60% sucrose was due to overloading of material onto the sucrose gradient. However, when only one-fifth as much sample was loaded, the same fractionation pattern was observed, and if material from the 60% sucrose fraction was re-applied to a second gradient, it was found to fractionate in the 60% layer (data not shown).

**Morphology of Assembled Vault-like Particles**—Analysis of the material fractionating in the 40 and 45% sucrose layers by electron microscopy (EM) revealed the presence of vault-like particles (VLPs) (Fig. 2, arrowheads). Although a considerable variation in particle morphology was observed, most VLPs were similar in size and shape to purified rat vaults (3). These particles varied from 32–37 nm in width to 59–65 nm in length.

*FIG. 1. Expression of rat MVP in Sf9 cells.* A, Sf9 cells, uninfected (lanes 1 and 2) and infected (lanes 3 and 4) with baculovirus carrying His-T7-tagged rat MVP (MVP) were fractionated into S100 (lanes 1 and 3) and P100 (lanes 2 and 4) (as described under “Results” and in Ref. 7). These fractions were resolved by 6% SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and immunostained with anti-MVP polyclonal antibodies. No immunoreactive band is seen in either fraction from uninfected cells, whereas an immunoreactive band of expected size for tagged MVP (~106 kDa) is seen in both of the fractions from infected cells. Molecular mass markers are indicated on the left in kDa. B, Sf9 cells infected with baculovirus containing His-T7-tagged rat MVP were subjected to a vault purification protocol as described under “Materials and Methods.” Equal portions of the sucrose gradient fractions (20, 30, 40, 45, 50, and 60%) were resolved by 7% SDS-polyacrylamide gel electrophoresis, and proteins were detected either by silver staining (top) or Western blot analysis (bottom) using monoclonal antibodies against the T7 epitope tag. Lanes 1–6 correspond to sucrose fractions 20–60%. Molecular mass markers are indicated on the left in kDa.
and displayed the distinctive bifold symmetric vault central barrel with dual caps (for a gallery of these structures see Fig. 3; see also top row, Fig. 3A, for a VLP with the barrel and caps labeled). The dimensions of these particles correlates very closely with negatively stained rat liver vaults examined with the same electron microscope. However, the negatively stained vaults and VLPs are about 15–20% smaller than unstained cryo-images of rat vaults, which have 42 × 75 nm dimensions (11). These differences are likely due to shrinking of the negatively stained particles during air-drying and some variation in the calibration of the two different microscopes used. The presence of these apparently “complete” structures was unexpected, as vaults from most mammalian species also comprise VPARP and TEP1, two proteins that have been predicted to form the vault cap (18) as well as the vault RNA. Although it is possible that certain insect proteins could substitute for the high molecular weight VPARP and TEP1 proteins, it is very likely that these complete particles comprise MVP alone. In *Dictyostelium*, vaults appear to be formed of two (and possibly three) highly related MVPs (19, 20). In addition, vaults isolated from *Top1*–deficient mice also have a typical vault morphology with a central barrel and caps on either ends (21). An examination of the most highly purified fraction of VLPs from MVP-expressing Sf9 cells (45% sucrose fraction) failed to detect any stoichiometric amounts of co-purified proteins (see Fig. 1B). Although these fractions are relatively homogenous with respect to protein composition (>90% silver-stained material is the expressed MVP), they are relatively heterogeneous with respect to structural variations. However, nearly all of the VLPs contained a distinctive cap, and therefore if one or two endogenous insect proteins were required for cap morphology they would likely be co-purified with MVP and observed in the silver-stained gels. The absence of distinct co-purified proteins, even ones at ~10% of the abundance of MVP, indicate that multiple copies of MVP are sufficient for formation of both the vault barrel and cap. It should be noted, however, that the caps seen in many of the VLPs appeared abnormal. Many were flatter than those seen in vaults, and some were misshapen and distorted (see Fig. 3A, arrowheads). A more definitive picture of VLP structure will require a cryoreconstruction of these particles. Nevertheless, it is still remarkable that multiple copies of a single protein can polymerize into a non-spherically or non-cylindrically symmetrical structure as complex as the vault.

Occasionally, VLPs were observed that appear to be flatter (with dimensions of ~40 × 70 nm). These structures, which are shown in Fig. 3B, resemble vaults generated *in vitro* from purified rat liver preparations treated with trypsin (3). These structures could be formed in the insect cells by endogenous proteases acting on MVP or because of proteolysis occurring during purification. There was no difference in the VLPs formed when either the vsvg or His-T7 tags were used on the rat MVP, suggesting that the ability of MVP to form these particles is not effected by the addition of the tag (data not shown). Taken together, these biochemical and EM studies show that the rat MVP contains sufficient information to direct assembly of vault-like particles with characteristics similar to purified endogenous rat vaults.

A close examination of the sucrose gradient fractions that contained expressed MVP (40, 45, 50, and 60% sucrose fractions) revealed various structures that appear to represent assembly intermediates and some higher order structures not previously observed in vault purifications. The assembly intermediates were open half-vaults that were present as partially “opened” barrels with the characteristic petals surrounding a central ring. These open half-vaults are seen occasionally in purified rat liver preparations and often in *Dictyostelium* vault preparations (1). A panel of these structures is shown in Fig. 4A. The larger VLP structures seen in the purified fractions are more difficult to explain. These ordered VLP polymeric structures (see Fig. 2, arrows), which we refer to as vaultimers, appear to contain three to more than a dozen half-VLPs (panels of these images are shown in Fig. 4, B–D). The most common vaultimer has four symmetrically arranged half-VLPs (see Fig. 4B). Some of the 4-ners (Fig. 4B, bottom) have a central density that could represent one or two additional half-VLPs protruding above and below the plane of the micrograph, indicating that the 6-mer could also be a common form. Although most of
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the half-VLPs in the vaultimers are capped, polymeric structures containing some uncapped and distorted capped VLPs were also observed. Although they were less abundant, larger vaultimers likely composed of 8 to >12 half-VLPs were also seen (Fig. 4D). As these larger structures appeared to be enriched at higher sucrose concentrations in the gradient, the mass of these structures likely affects their fractionation more than their density. Vaults have been purified from dozens of different species (1) and from different tissues, 2 yet to our knowledge these vaultimers have never before been observed. It is possible that they arise in baculovirus as a result of MVP over-expression, which could overwhelm the cellular assembly machinery, or factors that are required for complete particle assembly. Alternately, they might arise when vault particles lacking VPARP, TEP1, and vault RNA are assembled. Preliminary experiments aimed at disrupting vaultimers with high salt have thus far been unsuccessful and may indicate that, like vaults, these structures are highly stable.

In conclusion, the expression of rat MVP alone in Sf9 cells is sufficient to direct the assembly of vault-like particles. The fact that no endogenous Sf9 MVP or vaults could be detected supports the idea that these VLPs are formed solely from the expressed rat MVP. As stated previously, no MVP homologues have been identified in the Drosophila data base, and attempts to purify vaults from insects have been unsuccessful. However, it is possible that Sf9 cell proteins may interact weakly with the VLPs or in such low abundance, perhaps in a catalytic role, that they cannot be detected. In preliminary experiments (not shown), no vault-like particles were formed in vitro when MVP purified from MVP-expressing, urea-denatured Sf9 cell extracts was allowed to renature by dialysis, suggesting that cellular components might be required for assembly. Further work is now planned to identify such assembly factors. Coexpression studies with the other vault proteins, VPARP and TEP1, and with the vault RNA, are also planned to assess their role in particle morphology and VLP/vaultimer assembly.

Acknowledgments—We thank Dr. Phoebe Stewart (UCLA) for critical reading of the manuscript and helpful discussions.

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FIG. 4. Open half-VLPs and vaultimers formed in Sf9 cells. A, structures from the 45% sucrose fraction from His-tagged, MVP-expressing Sf9 cells reminiscent of half-vaults in an open conformation are displayed in a gallery of images. B, vaultimers, apparently composed of 4–6 half-VLPs from the 45 and 50% sucrose fractions from His-tagged, MVP-expressing Sf9 cells, are displayed in a gallery. The upper row represents 4-mers (i.e. four half-VLPs) without a central density. Vaultmers in the lower row have a central density that may indicate one or two half-VLPs projecting above or below the image plane (see Discussion). C, vaultimers that appear to be composed of five or more half-VLPs. D, vaultmers that appear to be composed of eight or more half-VLPs. These larger structures were found more often in the 50% sucrose fraction. In each of the panels, the bar represents 100 nm.