The \( M_r 193,000 \) Vault Protein Is Up-Regulated in Multidrug-resistant Cancer Cell Lines


Department of Pathology, Academic Hospital Vrije Universiteit, 1081 HV Amsterdam, the Netherlands [A. B. S., G. L. S., M. C. d. J., S. E. B., D. F. D., R. J. S.]; Department of Biological Chemistry, University of California at Los Angeles School of Medicine, Los Angeles, California 90095 [A. C. S., V. A. K., L. H. R.]; Department of Molecular Recognition, Institute for Animal Science and Health (ID-DLO), 8219 PH Lelystad, the Netherlands [J. W. S., R. H. M.]; and Institute for Hematology, Erasmus University Rotterdam, 3000 DR Rotterdam, the Netherlands [E. W.]

ABSTRACT

Vaults are 13 megadalton ribonucleoprotein particles composed largely of the major vault protein (MVP) and two high molecular weight proteins, p240 and p193, and a small vault RNA (vRNA). Increased levels of MVP expression, vault-associated vRNA, and vaults have been linked directly to multidrug resistance (MDR). To further define the putative role of vaults in MDR, we produced monoclonal antibodies against the \( M_r 193,000 \) vault protein and studied its expression levels in various multidrug-resistant cell lines. We find that, like MVP, p193 mRNA and protein levels are increased in various multidrug-resistant cell lines. Subcellular fractionation of vault particles revealed that vault-associated p193 levels are increased in multidrug-resistant cells as compared with the parental, drug-sensitive cells. Furthermore, protein analysis of postnuclear supernatants and coimmunoprecipitation studies show that drug-sensitive MVP-transfected tumor cells lack this up-regulation in vault-associated p193. Our observations indicate that vault formation is limited not only by the expression of the MVP but also by the expression or assembly of at least one of the other vault proteins.

INTRODUCTION

Resistance to a broad range of cytostatic drugs (MDR)\(^3\) can be mediated by Pgp or the MRP1. Pgp and MRP1 function as efflux pumps, decreasing intracellular drug accumulation (1). In clinical drug resistance, however, other mechanisms may play a role, e.g., involving drug sequestration into exocytotic vesicles. Evidence has been obtained that subcellular particles named vaults may play a critical role in such a mechanism (2, 3).

Vaults are 13 megadalton ribonucleoprotein particles containing three proteins of \( M_r 240,000, 193,000, \) and 110,000, respectively, and a small untranslated vRNA. A vault interacting protein of \( M_r 54,000 \) is occasionally observed in rat liver vault preparations (2). Vaults are widely distributed throughout eukaryotes, and their morphology is highly conserved among various species. The remarkable structural conservation and broad distribution of vaults suggest that their function is essential to eukaryotic organisms and that the structure of the particle must be important for its function (4–6). Although vault function is undetermined, it has been proposed that vaults may mediate transport of various substrates (7, 8). A role for vaults in intracellular traffic might be mediated by binding to cellular organelles through direct interaction with its targets (9). Recently, an interaction of vaults with intracellular steroid hormone receptors has been reported (10). Although the majority of vault particles are distributed throughout the cytosol, a portion of vaults has been localized to the nuclear membrane at or near the nuclear pore complex. Furthermore, the recent three-dimensional reconstruction of the vault particle reveals a hollow interior, which may prove important in the transport/sequestration of large substrates (11). On the basis of striking similarities between vault particle mass and symmetry and the predictive mass of the putative central plug of the nuclear pore complexes, a role of vaults in nucleocytoplasmic exchange has been proposed (12).

The discovery of a key role of VR-MDR in clinical drug resistance depended on the molecular identification of the LRP as the human MVP (3). LRP had been first identified in a non-small cell lung cancer cell line, selected in vitro for DOX resistance. The protein was subsequently found to be overexpressed in many human tumor cell lines characterized by their MDR phenotype, in the absence of drug accumulation defects such as mediated by Pgp (13). Moreover, LRP expression closely reflected known chemoresistance characteristics in broad panels of unselected tumor cell lines and untreated clinical cancers of different histogenetic origins (14, 15). Results from several, but not all, clinicopathological studies showed that LRP expression at diagnosis, rather than Pgp or MRP1 expression, is a strong and independent prognostic factor for poor response to chemotherapy and/or outcome, e.g., in ovarian carcinoma and leukemias (16). Most importantly, Kitazono et al. (17) demonstrated recently, using a LRP induction system and LRP-specific ribozymes, that LRP is involved in resistance to Adriamycin, vincristine, VP-16, Taxol, and gramicidin D and has an important role in the transport of Adriamycin between the nucleus and the cytoplasm in the SW-620 human colon carcinoma cell line. To avoid confusion, we will hereafter refer to LRP as MVP.

Studies on the role of vaults in MDR, including the cloning of the MVP cDNA, have thus far been based on polyclonal antisera and two mAbs, LRP-56 and LMR-5 (13, 18), directed against the MVP. To further define the role of vaults in MDR, the other components must be characterized. The human vRNA genes have been cloned, and within tumor cells, not all of the vRNA was found to be vault-associated. Sedimentation measurements of vault components in VR-MDR cell lines have revealed up to a 15-fold increase in vault copy number, coupled with a comparable shift of vRNA to the 100,000 \( \times g \) pellet, demonstrating that vault formation is limited by expression of MVP and/or one of the other vault proteins (19). Because MVP-transfected cells did not show a drug-resistant phenotype (3), the other vault components are thought to be essential for vaults to play a role in MDR.

Here we describe the production of the first mAbs against the \( M_r 193,000 \) vault protein. The p193 was recently identified by its interaction with the MVP in a yeast two-hybrid screen, and its identity was confirmed by peptide sequence analysis (20). Results from protein analysis of postnuclear supernatants and subcellular fractions, Northern analysis, immunocytochemical and coimmunoprecipitation studies show that: (a) p193 and MVP are both increased in various MDR cell lines and; (b) vault-associated p193 levels are up-regulated in these MDR cell lines but not in a drug-sensitive, MVP full-length, cDNA-transfected cell line, supporting the conclusion that functional
vault formation is limited by expression of MVP and expression of at least one of the other vault components, p193.

**MATERIALS AND METHODS**

**Cell Lines.** The following cell lines were used: the non-small cell lung carcinoma cell line SW-1573, its DOX-selected MDR variants SW-1573/2R120 (selected with 120 nM DOX) and SW1573/2R160 (160 nM; ref. 21), the small cell lung carcinoma cell line GLC4 and its DOX-selected partner GLC4/ADR (1152 nM; Ref. 22), the breast carcinoma cell line MCF7 and the corresponding DOX, and mitoxantrone-resistant selected cell lines MCF7/ D40 (400 nM DOX) and MCF-7/7MR (80 nM mitoxanthrone; Ref. 23), the parental ovarian carcinoma cell line A2780, and the MVP full-length, cDNA-transfected cell line AC16 (5). All cell lines were propagated in DMEM (Bio-Whittaker, Verviers Belgium) supplemented with 10% FCS (Integro, Zaandam, the Netherlands), penicillin (50 units/ml), and streptomycin (50 μg/ml) at 37°C in a humidified atmosphere containing 5% CO2. Cells were routinely tested to ensure the absence of Mycoplasma. The drug-selected cell lines were cultured in the presence of drugs every other week. The stable MVP cDNA transfectant was maintained in medium supplemented with 800 μg/ml G418 (Sigma Chemical Co., St. Louis, MO).

**Immunization and Generation of Hybridomas.** *Escherichia coli* BL21 (DE3) bacteria were transformed with the pET-28a (+) expression vector (Novagen, Madison, WI) containing a segment of p193 cDNA corresponding to amino acids 408–611 (20). The vector construct was induced to produce the His-tagged recombinant protein using isopropyl-β-D-thiogalactoside (0.33 mM; 4–6 h; 37°C). Total cellular lysate was used as immunization antigen. Female BALB/c mice (n = 4) received footpad injections of 25 μg of antigen emulsified in Freund’s incomplete adjuvant (Difco, Detroit, MI). Two booster injections (~18 μg of antigen without adjuvant) were given at 4- and 2-week intervals, respectively. Four days before fusion, a third booster injection (10 μg of antigen) was administered. Lymphocytes were isolated from the draining popliteal lymph nodes of the mice, mixed with mouse myeloma Sp2/0 cells in a ratio of 6:1, and fused using polyethylene glycol. The hybridoma supernatants were tested for the presence of antibodies against the immunization antigen or, as a control, 5 μg/ml of pET-28a (+) transformed *E. coli* BL21 (DE3) bacteria lysate without the segment of p193 cDNA corresponding to amino acids 408–611. The vector construct was induced to produce the His-tagged recombinant β-galactosidase protein. Hybridomas secreting antibodies of interest were subcloned three times by limiting dilution. Immunoglobulin subtypes of the selected mAbs were determined using an isotype reagent kit (Boehringer Mannheim, Indianapolis, IN).

**PEPSSCAN.** All overlapping dendecapeptides (12-mers) covering amino acids 408–611 of the p193 protein (beginning with the 12-mers 408–419, 409–420, and so forth) were synthesized and screened using the minipepscan method as described previously (24, 25). In credit card format of mini-PEPSSCAN cards (455 peptides/card), the binding of the anti-p193 mAbs to each peptide was tested in a PEPSSCAN-based ELISA. The 455-well credit card format of mini-PEPSSCAN cards (455 peptides/card), the binding of the anti-p193 mAbs to each peptide was tested in an ELISA. The 455-well credit card contains 12 wells per card, with each well containing a different 12-mer peptide. The mAbs were incubated with the peptide wells, and the binding was detected using peroxidase-conjugated anti-mouse IgG. The bound mAbs were detected using peroxidase-conjugated anti-mouse IgG.

**Screening, Cloning, and Isotyping.** After 9 days of growth in selective medium, the hybridoma supernatants were tested for the presence of antibodies of interest by ELISA. Plates (96-well) were coated with 5 μg/ml of the immunization antigen or, as a control, 5 μg/ml of pET-28a (+) transformed *E. coli* BL21 (DE3) bacterial lysate without the segment of p193 cDNA. The plates were incubated with mAbs at 37°C for 2 h, washed with PBS, and incubated with 1:1000 dilution of peroxidase-conjugated swine antimouse IgG1 (Dako; 1 h). The plates were washed with PBS and incubated with 1:5000 dilution of peroxidase-conjugated anti-mouse IgG. The plates were washed with PBS and incubated with 1:5000 dilution of peroxidase-conjugated anti-mouse IgG.

**MVP Antibodies.** MVP expression was studied using the rabbit polyclonal antibody Pab W. Pab W was raised as follows. A *Ncol-Env* fragment of 2631 bp (amino acids 1–871) was cloned between the *Ncol* and *EcoRI* sites of the pGEX-KG polylinker (26). The resulting glutathione S-transferase MVP fusion protein was expressed in *E. coli* DH5α as described by Guan and Dixon (26), except that the cells were lysed by sonication. The negatively charged glutathione S-transferase MVP fusion protein was bound to glutathione-Sepharose 4B beads (Pharmacia Biotechnology, Uppsala, Sweden), after which the MVP part was released by a thrombin digest. The fraction containing the MVP was concentrated by freeze-drying and used to immunize a rabbit. Also, MVP expression was studied with two MVP-specific murine mAbs (both of the IgG2b subclass) obtained in our laboratory: LRP-56, which was raised by immunization of mice with the MDR tumor cell line SW-1573/2R120 (13); and MVP-37, raised in mice against the above-described MVP construct.

**Immunocytochemistry.** Cytoskeleton preparations of tumor cell lines were air-dried, fixed at room temperature in acetone for 10 min or 3% (v/v) glutaraldehyde for 10 min. The paraformaldehyde-fixed cells were washed two times with PBS and then incubated with 20 mM glycine (pH 7.5) in PBS for 10 min to block unreacted aldehyde groups. This was followed by two washes in PBS/0.2% (w/v) BSA. Denaturation of intracellular proteins was done by applying 50 μl of 6% guanidine hydrochloride in 50 mM Tris-HCl (pH 7.5) to the cytoskeleton preparations for 10 min (27). The cells were then rinsed three times with PBS/0.2% BSA. All antibody dilutions were made in PBS/1% BSA. Between incubation steps, the acetone-fixed cytoskeleton preparations were washed (three times during 15 min) with PBS, paraformaldehyde-fixed preparations were washed with PBS/0.2% BSA. All antibodies were applied for 60 min at room temperature to the acetone-fixed cytoskeleton preparations and for 30 min at 37°C to the paraformaldehyde-fixed preparations. Irrelevant mouse IgG1 (Cappel, Organon Teknika, Aurora, OH) was used as negative control. Subsequently, the preparations were incubated with biotinylated rabbit antimouse Fab(′) fragments (Dako; 60 min at room temperature), followed by peroxidase-conjugated streptavidin (Zymed, San Francisco, CA; 30 min at room temperature). Bound peroxidase was visualized with 4 μg/ml amino-ethyl-carbazole and 0.02% (v/v) H2O2 in 0.1 M NaAc (pH 5.0), nuclei were counterstained with hematoxylin, and the cytoskeleton preparations were mounted with Kaiser’s mounting medium.

**Double Immunofluorescent Staining.** For double-labeling immunofluorescence experiments, cytoskeleton preparations were fixed in paraformaldehyde and pre-treated with glycine and guanidine hydrochloride as described above (see “Immunocytochemistry”). After a blocking step with 2% normal goat serum and 2% normal rabbit serum (Dako) for 20 min at room temperature, the cells were incubated simultaneously with anti-p193 (mAb p193-4) and anti-MVP (mAb MVP-37) for 30 min at 37°C. Subsequently, p193-4 was detected using biotinylated goat antimouse IgG1 (Southern Biotechnologies Inc., Birmingham, AL) with the addition of 10% human pooled serum and 10% normal goat serum, followed by the detection of MVP-37 with peroxidase-conjugated rabbit antimouse (Dako; 30 min/incubation). Biotinylated goat antimouse IgG1 was detected using streptavidin conjugated with R-phycocerrin (Dako) for 30 min, whereas rabbit antimouse peroxidase was detected by the deposition of FITC-conjugated tyramine (15 min; Refs. 28 and 29). The cytoskeleton preparations were counterstained with 4’,6-diamidino-2-phenylindole, mounted with Prolong mounting medium (Molecular Probes, Eugene, OR), and evaluated with a Leica DMRB fluorescence microscope. Negative controls consisted of simultaneously processed slides with isotype-matched control mAbs replacing each p193-4 or MVP-37 (mouse IgG1, Cappel; mouse IgG2b, anti-chromogranin A. Dako).

**Northern Analysis.** Total RNA was isolated following the procedure of Chomczynski and Sacchi (27). Total RNA (20 μg) was fractionated on a formaldehyde-agarose gel and transferred to Hybrid-N membrane (Amersham Corp., Little Chalfont, United Kingdom). Hybridization was carried out according to the manufacturer’s recommendation with a randomly primed p193 probe (bases 4515–5490). Hybridized bands were visualized on a PhosphorImager screen (Molecular Dynamics).

**Protein Analysis of Postnuclear Supernatant.** Extracts were prepared from various drug-sensitive, resistant, and revertant cell lines by the following procedure. Cells were harvested and resuspended in cold buffer A [50 mM TrisCl (pH 7.4), 1.5 mM MgCl2, and 75 mM NaCl] containing 0.5% NP40 and 1 pH phenylmethylsulfon fluoride. All subsequent steps were performed at 4°C. Samples were vortexed, sonicated on ice for 5 min, and centrifuged at 9000 × g for 20 min. The resulting supernatant was designated as the postnuclear supernatant. Protein concentration was determined with a Bio-Rad protein assay (Bio-Rad, Richmond, CA). Protein samples were fractionated by SDS/6% PAGE and subsequently transferred to nitrocellulose filter by electrobetting. After blotting, the filters were blocked for at least 2 h in block buffer (PBS containing 1% BSA, 1% milk powder, and 0.05% Tween 20), followed by overnight incubation with the primary antibodies in block buffer/10% FCS. Immunoreactivity was visualized with peroxidase-conjugated swine antirabbit or rabbit antimouse immunoglobulins (Dako) in block buffer/10% FCS, followed by staining with 0.05% chloroform and 0.03% H2O2 in PBS. Protein levels were determined by densitometric scanning (GelDoc; Bio-Rad).
RESULTS

Generation of mAbs against p193. Using popliteal lymph nodes from mice immunized with an *E. coli* lysate transformed with the pET28a(+) expression vector containing amino acids 408–611 of the p193 cDNA, murine hybridomas were generated and screened for their ability to detect the immunization antigen by ELISA. Three stable, cloned hybridoma cell lines, designated p193-4, p193-6, and p193-10 were obtained. mAb p193-4 was determined to be of the IgG2a subclass, mAb p193-6 of the IgG2b subclass, and mAb p193-10 was an IgG2a subclass, mAb p193-6 FSKVEDY (amino acids 593–599), and by mAb p193-10 VALGK (amino acids 506–510).

Coordinate Expression of p193 and MVP in Tumor Cell Lines. Protein analysis of postnuclear supernatants with the mAbs p193-4, p193-6, and p193-10 show immunoreactivity with, respectively, dodecapeptide 76–84, 181–188, and 92–99. The core epitope recognized by mAb p193-4 is HPGE (amino acids 491–494), by mAb p193-6 FSKVEDY (amino acids 593–599), and by mAb p193-10 VALGK (amino acids 506–510). Similar trend in up-regulation as the MVP protein. The parental drug-sensitive GLC4, SW-1573 and MCF-7 (breast carcinoma cell line) and the multidrug resistant SW-1573/2R160 and MCF-7/D40 cell lines analyzed with no or only low levels of MVP also lacked detectable p193 protein (Fig. 2). Densitometric analysis confirmed the observed coordinate expression of p193 and MVP in the panel of cell lines GLC4, GLC4/ADR, SW-1573, SW-1573/2R120, SW-1573/2R160, MCF-7, and MCF-7/D40, as indicated by the *A* × mm² values of p193 (including lower protein bands) and MVP bands: 0.4, 10.1, 1.5, 11.9, 1.2, 0.3, 0.6 and 0, 3.7, 1.1, 3.4, 0.2, 0, 1.4, respectively.

Northern analysis of total RNA from the parental GLC4 cell line and its drug-resistant, derivative cell line GLC4/ADR revealed that p193 mRNA levels are increased in the GLC4/ADR cell line, in conjunction with the observed increased expression of p193 protein. In the drug revertant GLC4/REV line, which was isolated by culturing the cells in the absence of drug, resulting in a much less drug-resistant cell line, the p193 mRNA level was intermediate (Fig. 3). Thus, both p193 mRNA and protein expression are regulated according to drug resistance level, concomitant with MVP mRNA and protein levels (3, 19, 31).

Immunocytochemical staining of cytocentrifuge preparations confirmed the correlation between p193 and MVP expression in the tumor cell lines. Although less intense, the granular p193 staining throughout the cytoplasm of drug-resistant GLC4/ADR (Fig. 4b) and SW-1573/2R120 cells (Fig. 4d) is similar to that observed by MVP staining (Fig. 4, f and h, respectively). No expression of p193 was observed with mAb p193-4 and p193-10 (not shown). The expression of the p193 protein in these cell lines, as well as lower molecular weight bands, most likely reflecting breakdown products, shows a similar trend in up-regulation as the MVP protein. The parental drug-sensitive GLC4, SW-1573 and MCF-7 (breast carcinoma cell line) and the multidrug resistant SW-1573/2R160 and MCF-7/D40 cell lines analyzed with no or only low levels of MVP also lacked detectable p193 protein (Fig. 2). Densitometric analysis confirmed the observed coordinate expression of p193 and MVP in the panel of cell lines GLC4, GLC4/ADR, SW-1573, SW-1573/2R120, SW-1573/ 2R160, MCF-7, and MCF-7/D40, as indicated by the A × mm² values of p193 (including lower protein bands) and MVP bands: 0.4, 10.1, 1.5, 11.9, 1.2, 0.3, 0.6 and 0, 3.7, 1.1, 3.4, 0.2, 0, 1.4, respectively.

Northern analysis of total RNA from the parental GLC4 cell line and its drug-resistant, derivative cell line GLC4/ADR revealed that p193 mRNA levels are increased in the GLC4/ADR cell line, in conjunction with the observed increased expression of p193 protein. In the drug revertant GLC4/REV line, which was isolated by culturing the cells in the absence of drug, resulting in a much less drug-resistant cell line, the p193 mRNA level was intermediate (Fig. 3). Thus, both p193 mRNA and protein expression are regulated according to drug resistance level, concomitant with MVP mRNA and protein levels (3, 19, 31).

Immunocytochemical staining of cytocentrifuge preparations confirmed the correlation between p193 and MVP expression in the tumor cell lines. Although less intense, the granular p193 staining throughout the cytoplasm of drug-resistant GLC4/ADR (Fig. 4b) and SW-1573/2R120 cells (Fig. 4d) is similar to that observed by MVP staining (Fig. 4, f and h, respectively). No expression of p193 was
detected in the parental drug-sensitive GLC4/S (Fig. 4a) and SW-1573 (Fig. 4c) cell lines, which show no and only weak expression of MVP (Fig. 4, e and g, respectively).

**Overlapping Distribution of p193 and MVP in Tumor Cell Lines.** By double immunofluorescence labeling experiments, we compared the subcellular localization of the p193 protein with that of the MVP protein. Double labeling of vault-overexpressing GLC4/ADR cells using mAb p193-4 against p193 and mAb MVP-37 against MVP revealed an identical cytoplasmic granular staining pattern of both vault proteins (Fig. 5, a–c). Similarly, colocalization of p193 and MVP distribution was found in the vault-positive SW-1573/2R120 cells (data not shown). As described previously (13), only 1–3% of the cells from the SW-1573/2R160 cell line stained positive for MVP (Fig. 5e). These cells also showed p193 (Fig. 5d) staining, which completely colocalized with the MVP (Fig. 5f). No R-phycocerythrin/p193 labeling was observed when irrelevant mouse IgG1 (Cappel) replaced p193-4. No FITC/MVP labeling was observed when irrelevant mouse IgG2b (anti-chromogranin A; Dako) replaced MVP-37.

**Up-Regulation of Vault-associated p193 in MDR Tumor Cells.** By subcellular fractionation, Kedersha and Rome (2) showed that vaults pellet at 100,000 × g (P100), and that all of the MVP is associated with this fraction and is assembled into vaults. In contrast, only a portion of the total cellular vRNA fractionates to the P100, where it is associated with vaults. The non-vault-associated vRNA fractionates in the soluble or S100 fraction (19). Here, we determined the p193 levels in these fractions of the parental, drug-sensitive GLC4 cell line and its derivative cell lines, the drug-resistant GLC4/ADR and drug revertant GLC4/REV (Fig. 6). Unlike the MVP protein, the p193 protein was observed in all of the fractions: N, S100, and P100; however, the greater part fractionated with the P100. The detection of p193 in the S100 fraction indicates that besides vault-associated p193 also non-vault-associated p193 is present. Furthermore, a comparison of parental, resistant, and revertant cells revealed that a higher amount of p193 fractionates with the P100 of the MDR GLC4/ADR cell line. Although less obvious, the N and S100 fractions of these drug-resistant cells also contained increased levels of p193 protein as compared with the parental and revertant cells. Analysis of additional MDR lines, including the parental, drug-sensitive lines (SW-1573, SW-1573/2R120, MCF-7, and MCF-7/MR; data not shown) confirmed our observation that in MDR cells clearly a higher amount of the p193 is localized in the P100, the fraction that reflects the level of assembled vault particles.

To further analyze the association of the p193 with assembled vault particles, we immunoisolated vault particles from the postnuclear supernatant of the multidrug-resistant GLC4/ADR cell line using the anti-MVP mAb LRP-56. The precipitated structures were negatively stained and examined by electron microscopy. The ovoid structures that were revealed have similar dimensions to those seen in purified vault samples described previously (Fig. 7; Refs. 2, 4–6).
distortion of the vault morphology is probably attributable to the presence of the bound antibodies on the surface of the vault particle or disruption of the particle from antibody interactions. No vault structures were detected in control immunoprecipitates (not shown). Immunoprecipitation of the MVP/vaults from postnuclear supernatant of the multidrug-resistant GLC4/ADR cell line, followed by detection of MVP as well as p193 by immunoblotting experiments, showed clear coimmunoprecipitation of the p193 with the MVP (Fig. 8). Control immunoprecipitation with an isotypic mAb revealed neither MVP nor coimmunoprecipitation of p193. Both proteins were localized in the corresponding supernatant. In contrast with our fractionation data, no soluble p193 was detected in the supernatant that remained after MVP precipitation. This is not surprising because the supernatants that were examined are much less concentrated than the immunoprecipitation samples. As a result, the relatively low levels of non-vault-associated p193 may remain below the level of detection.

**Lack of p193 Expression in MVP-transfected, Drug-sensitive Cells.** We have shown previously by transfection studies that overexpression of the MVP cDNA alone is not sufficient to confer a drug-resistant phenotype. Because vaults are multisubunit particles, we reasoned that additional components could be required for functional vault formation (3). To examine the possibility that the assembly of functional vault particles is limited by p193 expression, we determined the level of p193 expression in the MVP-transfectant cell line AC16, which was derived from the parental, drug-sensitive A2780 ovarian carcinoma cell line (3). The GLC4/ADR cell line was included as a positive control. As analyzed by Western blotting, the AC16 cells clearly overexpress MVP as compared with the parental A2780 cells. However, a comparison of the p193 expression levels in the MVP-transfected and the parental cell line revealed no concomitant induction of p193 expression in the MVP transfectant (Fig. 9). Furthermore, immunoprecipitation of the MVP from the postnuclear supernatant of the AC16, followed by SDS-PAGE analysis, showed only detection of MVP (Fig. 8).

**DISCUSSION**

There is still much speculation about the function of vaults. As an apparently ubiquitous and highly conserved subcellular particle (4-6, 1108).

---

**Fig. 5.** Overlapping distribution of p193 and MVP in tumor cells. Double staining of p193 with mAb p193-4 (~5 μg/ml) and MVP with mAb MVP-37 (~15 μg/ml) in GLC4/ADR (a–c) and SW-1573/2R160 cells (d–f) is shown. a and d, p193 (red) visualized by R-phycocerythrin-labeled streptavidin. b and e, green staining (FITC-conjugated tyramine) indicates MVP expression. c and f, p193/MVP double staining as detected using a triple filter, simultaneously visualizing colocalization of p193 and MVP (yellow) and counterstained nuclei (blue).

**Fig. 6.** Vault-associated p193 (P100) levels are up-regulated in GLC4/ADR cells. p193 levels were determined by Western blotting (using mAb p193-4 ~20 μg/ml) in subcellular fractions as described in “Materials and Methods.” Sample extracts for each cell line are in groups of three: nuclear (N), high-speed supernatant (S), and high-speed pellet (P) from the parental GLC4, resistant GLC4/ADR, and revertant GLC4/REV.

---

**Fig. 7.** Electron microscopy of negatively stained vaults obtained by immunoprecipitation of MVP using mAb LRP-56 from GLC4/ADR cells. Four vault particles from one immunoprecipitation experiment are shown.
vaults, must have an important function in fundamental cell processes. On the basis of structure analyses and subcellular localization studies, it has been postulated that they mediate nucleocytoplasmic exchange as well as vesicular transport of various substrates, including cytosolic drugs. In support of this view, the clinical relevance of vaults in the prediction of a multidrug-resistant phenotype in numerous cancer cell types is well documented (14–16). Recently, Kitazono et al. (17) showed that reduction of MVP expression by use of MVP-specific ribozymes in a cell line induced to overexpress MVP by exposing cells to sodium butyrate was enough to prevent drug resistance. Furthermore, the nuclei isolated from sodium butyrate untreated cells or those from treated cells in the presence of anti-MVP antibodies accumulated cytostatic drugs, but those from treated cells in the absence of antibodies did not. These findings provide the first causal relationship between MVP expression and drug resistance.

Studies on the function and structure of vaults to date have been focused on the MVP and vRNA. In this study, we generated mAbs against the p193 and used these to further characterize the p193 in human tumor cell lines. A strong positive correlation was found between p193 and MVP expression by Western analysis, suggesting that besides MVP also p193 expression is indicative for VR-MDR. In addition, analysis of total RNA indicated that besides the p193 protein also p193 mRNA levels increase accordingly to an increase in the number of vaults. Immunocytochemical analysis showed that the distribution of p193 staining is compatible with the cytoplasmic location of MVP/vaults. Parafomaldehyde fixation followed by a guanidine hydrochloride denaturation pretreatment (27) is a prerequisite for exposing the epitopes recognized by the anti-p193 mAbs. This protocol could not retrieve the antigenic site for the anti-MVP mAb LRP-56, which gives a positive signal in cells fixed with acetone. This is not surprising because the anti-p193 mAbs were raised against a fusion protein and therefore are more likely to be unreactive with their antigens in a more native conformation than the anti-MVP mAb LRP-56, which was raised against tumor cell lysate.

Comparison of the distribution of the p193 with the MVP protein in tumor cells reported earlier to contain high amounts of vault particles (19) reveals an identical staining pattern in corresponding immunofluorescent images. Typical vault granules are present in the entire cytoplasm, which colocalize in the double-exposed image. The double staining results strongly suggest a high degree of association of the p193 with MVP/vaults. On the basis of MVP overexpression, vaults have been reported to be most abundant in epithelial cells (5, 15). Although the precise tissue distribution of the p193 is still under investigation, we found p193 to be present in normal human lung, with highest expression in the epithelial cells lining the respiratory tract. Thus, vault expression in normal human tissues also involves p193 expression.

Upon subcellular fractionation, the MVP is exclusively present in the large vault complex retained in a particulate fraction (2, 5, 19, 32). Western analysis of similar subcellular fractions of tumor cell lines demonstrates the presence of vault-associated as well as a relatively low level of non-vault-associated p193. In a previously reported study, (20) the presence of non-vault-associated p193 was also observed in the cytosol and nucleus by Western blot analysis upon subcellular fractionation. In contrast with the present findings, the nuclear localization of p193 was also shown in these studies by immunofluorescence using an anti-p193 polyclonal antibody. Probably the relatively low level of non-vault-associated p193 in the drug-resistant cell lines remains below the detection level in our immunofluorescence double-labeling experiments using anti-p193 mAbs, or the soluble p193 signal is masked by the abundance of MVP located in the cytoplasm. A comparison upon subcellular fractionation of resistant and parental tumor cell lines reveals a clear increase in vault-associated p193 levels in the resistant cells. Furthermore, in the revertant GLC4 cell line, which was isolated by culturing in the

---

**Fig. 8.** Coimmunoprecipitation of p193 with MVP in multidrug-resistant GLC4/ADR cells, not in drug-sensitive AC16 and parental A2780 cells. Western analysis of p193 (using the mAb p193-4; 15 μg/ml) and MVP (using the Pab W 1:500) in MVP immunoprecipitates (using the mAb LRP-56) and isotypic, control immunoprecipitates and the supernatants of these immunoprecipitates. The precipitations were carried out on postnuclear supernatants of the cell lines A2780, AC16, and GLC4/ADR.

**Fig. 9.** Lack of p193 expression in AC16 cells. Western analysis of p193 (mAb p193-4, 15 μg/ml) and MVP (Pab W 1:500) levels in 80 μg of postnuclear supernatants from the multidrug-resistant non-small cell lung cancer cell line GLC4/ADR, the drug-sensitive ovarian cancer cell line A2780 and its derivative, the drug-sensitive full-length MVP cDNA transfectant AC16.
absence of drug but still is a drug-resistant cell line (albeit at a lower concentration of drug), vault-associated p193 protein levels decrease. Thus, an increase in the general pool of vaults (as in the drug resistant cells) results in an increase in vault-associated p193.

To further evaluate the association of p193 with MVP/vaults, we performed immunosioination of vault particles from GLC4/ADR cells. Negative staining electron microscopy showed that vault particles were isolated as judged by the structure resemblance to vaults described previously (2, 5, 6). Western analysis of the immunosolates revealed coimmunoprecipitation of the p193 with the MVP, demonstrating the specificity of p193 association with MVP/vaults.

Consistently, vault formation is limited by the expression of MVP (19). However, the previously constructed MVP transfected tumor cell line AC16 shows no signs of drug resistance (3). This is not unexpected because the MVP comprises only 70% of the vault particle. Therefore, additional components of the vault particle could also be required for vault function and drug resistance. Within tumor cells, vRNA was found to be in considerable excess to MVP (19), suggesting that in the AC16 cells, this component is not limiting functional vault formation. Western analysis revealed that endogenous p193 was not detected as a component in the telomerase complex (telomerase-associated particle). Therefore, additional components of the vault particle could also be required for vault function and drug resistance.

Taken together, using newly developed mAbs against the p193, we have demonstrated that the MVP and p193 are co-up-regulated in various MDR cell lines. Furthermore, we present evidence that functional vault particles is impaired by the lack of overexpression of at least another vault constituent and subsequent vault association, leading to high numbers of incomplete dysfunctional vault particles.

ACKNOWLEDGMENTS

The technical assistance of Marcel B. H. J. Vervoort, Wim Vos, Jan M. Fritz, and Harold H. J. Backus is gratefully acknowledged.

REFERENCES

The $M_r$ 193,000 Vault Protein Is Up-Regulated in Multidrug-resistant Cancer Cell Lines

Anouk B. Schroeijers, Amara C. Siva, George L. Scheffer, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/60/4/1104

Cited articles
This article cites 31 articles, 13 of which you can access for free at:
http://cancerres.aacrjournals.org/content/60/4/1104.full.html#ref-list-1

Citing articles
This article has been cited by 9 HighWire-hosted articles. Access the articles at:
/content/60/4/1104.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.