Vaults Are Up-regulated in Multidrug-resistant Cancer Cell Lines*

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 Vaults are large, oval-shaped, cytoplasmic ribonucleoprotein particles originally identified in preparations of clathrin coated vesicles (10–12). Purified vaults are isolated from the microsomal fraction (100,000 × g pellet; P100) by a series of sucrose gradients followed by electrophoresis on nondenaturing agarose gels (10, 13). Vaults purified from rat liver consist of three protein species (210, 192, and 104 kDa) and a small RNA, vRNA. The vRNA constitutes 5% of the rat vault particle by mass and is not a structural component, as ribonuclease digestion does not alter particle morphology (14). vRNAs isolated from different species share a similar predicted secondary structure despite their differences in length, indicating that vRNA association with vaults is not fortuitous (15). This suggests that it has a fundamental role in the function of the vault particle possibly through RNA-RNA or RNA-protein interactions. The 104-kDa MVP constitutes >70% of the total protein from rat vaults and is the main structural component of the particle. Although the size of the MVP varies slightly among species, it retains immunological cross-reactivity (16). Vaults are widely distributed throughout eukaryotes, and their morphology is highly conserved among these species. The structure of the vault particle has been extensively studied by electron microscopy (14). Its dimensions have been determined to be 55 × 30 nm, with a molecular mass of about 13 MDa (three times the size of a ribosome). The intact vault particle has 2-fold symmetry, with each half vault capable of opening into a flower-like structure containing eight petals surrounding a central ring. The remarkable 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. Although vault function is undetermined, a portion of vaults have been localized to the cytoplasmic face of the nuclear membrane at or near nuclear pore complexes (17). Moreover, vault particle mass and symmetry are strikingly similar to the predicted mass of the putative central plug of the nuclear pore complexes (17). Vault particle mass and symmetry are strikingly similar to the predicted mass of the putative central plug of the nuclear pore complexes, leading us to propose that vaults participate in nuclear-cytoplasmic transport. This hypothesis has increased in significance in view of the finding that MVP is up-regulated in non-Pgp MDR cell lines.

Here we describe the cloning of the human vRNA genes and show by subcellular fractionation of vault particles that both MVP and vault-associated vRNA levels are similarly increased in various non-Pgp drug-resistant cell lines. These results support the conclusion that vaults are up-regulated in certain drug-resistant cell lines.

EXPERIMENTAL PROCEDURES

Cell Line Maintenance—MDR cell lines, SW1573/2R120 (non-small cell lung cancer) (18), and GLC4/ADR (small cell lung cancer) (19, 20), were cultured in the presence of 115 and 1156 nM doxorubicin, respectively, once every week. MCF-7/1R (breast cancer cells) (21) and 8226/MR4, 8226/MR20 (myeloma cells) (22) were cultured in the presence of 80, 40, and 200 nM mitoxanthrone, respectively, twice weekly. Drug-
sensitive SW1573, GLC4/S, MCF-7, and 8226/S cells were grown in RPMI 1640 medium, supplemented with 10% fetal bovine serum and antibiotics.

**Human vRNA Cloning**—To isolate the genes encoding the human vault vRNA genes we screened a Lambda FIX II (Stratagene) human genomic DNA library that was constructed by Kathy Kampf (UCLA MRRC Molecular Biology Core Facility). A total of 6.25 × 10⁶ recombinants were screened as described previously (15) using a random primed partial human vRNA gene. Comparisons between the rat and bullfrog vRNA sequences revealed that bases 11–27 and 110–129 (based on the rat vRNA sequence) were conserved (15). Primers to these conserved regions were synthesized on an Applied Biosystems DNA synthesizer and used to amplify partial human vRNA genes by polymerase chain reaction using DNA-aided cDNA synthesis. The PCR products were subcloned into pBluescript SK+ (Stratagene) and sequenced by the dyeoxy method using Sequenase (U. S. Biochemical Corp.). These partial human vRNA genes were used as hybridization probes. Two clones were identified and plaque-purified (1 and 4). Based on Southern blot analysis a 350-base pair fragment was subcloned into pBluescript SK+ (HVG1) and sequenced. Subcloning and sequence analysis revealed that the second clone (no. 4) contained two vRNA genes (HVG2 and HVG3) within about 7 kilobase pairs of each other. The sequences have been submitted to GenBank™ (accession nos. AF045143, AF045144, and AF045145; HVG1 through HVG3, respectively).

**Subcellular Fractionation**—Extracts were prepared from various drug-sensitive, -resistant, and -revertant cell lines by the following procedure. Cells (10⁶) were harvested, counted, and resuspended in 5 ml of cold buffer A (50 mM Tris-Cl (pH 7.4), 1.5 mM MgCl₂, 75 mM NaCl) containing 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitor mixture (2 μg/ml aprotinin, 0.5 mM benzamidine, 2 μg/ml chymostatin, 5 μg leupeptin, 5 μg pepstatin). All subsequent steps were performed at 4°C. Cells were vortexed, incubated on ice for 5 min, and centrifuged at 20,000 × g for 20 min. The postnuclear supernatant fraction was centrifuged at 100,000 × g for 1 h. The resulting supernatant was designated the S100 fraction. The nuclear layer were collected, diluted 4-fold with buffer A, and centrifuged for 3 h on a SW-41 rotor for 16 h. Under these conditions, intact vault particles (like the minor vault proteins and vRNA) could also be required for drug resistance. Partial sequence conservation between rat and bullfrog vRNAs (15) (data not shown). Like other vRNA genes, the human vRNA fractionates in the soluble or S100 fraction (Fig. 2A).

**RESULTS AND DISCUSSION**

MVP has been shown to be overexpressed in many non-Pgp MDR tumor cell lines, including the SW1573/2R120 (non-small cell lung cancer) (18), GLC4/ADR (small cell lung cancer) (19, 20), MCF-7/MR (breast cancer) (21), and 8226/MR20 (myeloma) (22) cell lines (6). Furthermore, revertant cell lines, which were isolated by culturing in the absence of drug, down-regulate the expression of MVP (6). However, transfection studies have shown that overexpression of the MVP cDNA alone is not sufficient to confer a drug resistant phenotype (9). This is not unexpected as the MVP comprises only 70% of the vault particle mass. Therefore additional components of the vault particle (like the minor vault proteins and vRNA) could also be required for drug resistance. Partial sequence conservation between rat and bullfrog vRNAs allowed us to clone the human vRNA genes. Humans contain three vRNA homologues that share about 84% identity with each other: one is 96 bases in length (human vRNA gene, HVG1) and the other two are 86 bases in length (HVG2 and HVG3; Fig. 1). The human vRNAs can be folded into secondary structures similar to those for rat and bullfrog vRNAs (15) (data not shown). Like other vRNA genes, the human vRNA genes contain internal RNA polymerase III-type promoter elements and end with a typical polymerase III termination signal of four Ts (Fig. 1). We have previously shown, by subcellular fractionation, that vaults pellet at 100,000 × g (P100) and that all of the MVP is associated with this fraction and is assembled into vaults (10). In contrast, only a portion of the total cellular vRNA fractionates to the P100 where it is associated with vaults. This non-vault-associated vRNA fractionates in the soluble or S100 fraction (Fig. 2A).

Although there are multiple human vRNAs, we have determined that only one form (HVG1) associates with the vault
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Experimental Procedures.

GLC4/REV).

line GLC4, parental (GLC4/S), resistant (GLC4/ADR), and revertant (GLC4/REV). B, Northern analysis of RNA extracted from fractions (see “Experimental Procedures”). C, Northern analysis of 10 μg of total RNA.

Fig. 3. A, MVP levels were determined by Western blotting. Sample extracts for each cell line are in groups of three: nuclear (N), high speed supernatant (S), and high speed pellet (P) from the non-small cell lung cell line GLC4, parental (GLC4/S), resistant (GLC4/ADR), and revertant (GLC4/REV). B, Northern analysis of RNA extracted from fractions (see "Experimental Procedures`). C, Northern analysis of 10 μg of total RNA.

TABLE I

Comparison of MVP and vRNA levels in drug-resistant lines

| Drug-resistant | P100 | Fold change |
|---------------|------|-------------|
| GLC4/adr      | MVP  | 15.1        |
|                | vRNA | 13.7        |
| GLC4/rev      | MVP  | 4.4         |
|                | vRNA | 4.6         |
| SW1573/2R120  | MVP  | 2.3         |
|                | vRNA | 3.6         |
| 2R120/rev     | MVP  | 1.5         |
|                | vRNA | 1.2         |
| 8226/4R20     | MVP  | 1.5         |
|                | vRNA | 1.5         |
| 8226/6R20     | MVP  | 1.3         |
|                | vRNA | 1.6         |
| MCF7/8        | MVP  | 3.7         |
|                | vRNA | 2.9         |

* The P100 protein and RNA levels were compared in drug-resistant and parental cell lines to determine the fold increase (drug-resistant/parental = fold change).

particle (as evidenced by pelleting at 100,000 × g, Fig. 2A). Surprisingly, the 86-bp encoded vRNAs are not present in all of the cell lines (Fig. 2B, lanes 1–3, 9, and 10). However, PCR analysis of genomic DNA from these lines indicates the genes are present (data not shown). These data indicate that, while the 86-bp RNAs (HVG2 and HVG3) are vRNA-related, they are apparently not vault-associated RNAs.

Previous studies of non-Pgp MDR cells have determined that MVP levels are increased, but the levels of vault particles has not been investigated (6, 9). Here we measured vault particle levels by sedimentation analysis in conjunction with an analysis of the levels of MVP and associated HVG1 vRNA in the small cell lung cancer cell line GLC4/S (parental), and its derivative cell lines the drug resistant GLC4/ADR and drug revertant GLC4/REV (Fig. 3, Table I). A comparison of the resistant and parental MVP revealed a 15-fold increase in MVP protein levels and suggested that the increased protein was assembled into a macromolecular form able to pellet at 100,000 × g (Fig. 3A, lanes 3 and 6). This 15-fold increase in MVP protein levels is in agreement with the increased expression of MVP mRNA (9). In addition, Northern analysis of the same fractions extracted for RNA revealed that vault-associated HVG1 vRNA increased about 15-fold (Fig. 3B, lanes 3 and 6). A concomitant shift of the vRNA from the S100 to the P100 fraction in the drug resistant line (Fig. 3B, lanes 3 and 6) suggests that a larger fraction of vRNA is associated with vaults. Likewise in the revertant line, which was isolated by culturing in the absence of drug, but is still a drug-resistant cell line (albeit at a lower concentration of drug), protein and vRNA levels decrease (Fig. 3A, lanes 6 and 9; Fig. 3B, lanes 6 and 9). Both the MVP and vRNA levels decrease to comparable levels in the revertant line (Table I).

Analysis of total RNA indicates that vRNA levels remain constant in the different lines, and that increased association with vaults is not due to an increase in transcription of the vRNA gene (Fig. 3C). This is not surprising since we have determined that on average only about 20% of the total vRNA is associated with the vault particle in these MDR cancer cell lines (data not shown). These results support the hypothesis that there is a dynamic relationship between the vRNA and vaults and that there is a pool of vRNA from which a certain fraction is associated with the vault particle at any particular time. Thus an increase in the general pool of vaults (as in the drug resistant lines) results in an increase in the fraction of the vRNA pool that is vault-associated (Fig. 3B, lanes 5 and 6). Correspondingly, in the revertant lines, a shift of vRNA from the P100 back to the S100 pool is observed (Fig. 3B, lanes 5 and 6), supporting our view that the vRNA is a dynamic component of the vault particle. Recent studies in our laboratory have

Fig. 4. A Western analysis of MVP levels in a sucrose equilibrium gradient. Fractions correspond to the load 20, 30, 40, 45, 50, and 60% layers (lanes 1–6, respectively); lane 7, GLC4/ADR P100; lane M is the Novagen Perfect Protein Marker. B, Northern analysis of RNA extracted from gradient fractions (lanes 1–6) and a total RNA (SW1573) standard (lane 7).

Fig. 5. Determination of vault copy number in parental cell lines. P100 fractions representing 2 × 105 or 4 × 105 cells per lane from the parental cell lines (lanes 1, 2, and 3, respectively) and purified rat liver vaults (0.05–0.25 μg per lane as indicated, lanes 5–9) were analyzed by Western blotting.

TABLE II

Estimation of vault particles

| Cell line   | Cancer type | Parental vaults/cell (×103) | Drug-resistant vaults/cell (×103) | Revertant vaults/cell (×105) |
|-------------|-------------|----------------------------|---------------------------------|-----------------------------|
| GLC4/S      | Small cell lung | 16 ± 0.7                  | 245                              | 71                          |
| SW1573/S    | Non-small cell lung | 37 ± 3.9                  | 85                              | 56                          |
| 8226/S      | Myeloma     | 35 ± 1.4                  | 46 (MR20)                        | NA                          |
| MCF7/8      | Breast      | 11 ± 0.4                  | 39                              | NA                          |

* These cells revert from the high level of drug that the resistant line can grow in, but are still resistant to a lower level of drug. NA, not available.

a Calculated using the number of vaults/cell measured in the parental cell lines times the fold changes determined in Table I.
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demonstrated by UV cross-linking that the vRNA interacts primarily with the minor vault proteins and not the MVP. Further fractionation of the GLC4/ADR P100 fraction on a sucrose equilibrium gradient revealed that the majority of MVP and vRNA were present in the 45% layer, coincident with the previously published behavior of purified vault particles (Fig. 4). This data verifies that the increases of MVP and vRNA seen in the P100 fraction accurately reflect the level of assembled vault particles. From these data we conclude that the co-ordinate changes in MVP and vRNA levels in the P100 fraction indicate that vault particle levels vary depending on the level of drug susceptibility in the cell lines.

We extended this analysis to four additional MDR lines including the parental and when available to revertant line(s). The results are summarized in Table I which shows the fold change in the relative levels of P100 MVP and vRNA compared with their parental cell lines. This data supports the conclusion that vault particle levels are also up-regulated in these MDR cell lines. Analysis of Table I revealed that as little as 1.3-fold up-regulation was sufficient to confer the drug resistant phenotype in one cell line compared with a high of 15-fold seen in another. We reasoned that this variability might be explained by a difference in the number of vault particles present in the parental cell lines, perhaps a higher induction would be necessary if the parent cell started with a lower level of endogenous vaults. Vault particle levels were determined by analysis of protein levels in the P100 fraction compared with increasing amounts of purified vaults (Fig. 5). As summarized in Table II, vault levels per cell vary considerably among the different cell lines examined and, consistent with our hypothesis, lines with lower levels of endogenous vault particles (GLC4/S and MCF7/S) showed the highest fold induction seen in Table I. Among the lines described in Table II, two (GLC4 and SW1573) were selected with doxorubicin (18–21), the others were selected with mitoxanthrone (21, 22). The very high number of vaults in the GLC4 drug resistant line (245,000 vaults per cell) might reflect the high concentration of doxorubicin to which this line is resistant (1 μM), this level of doxorubicin is ten times greater that the level used to select the SW1573 resistant lines 0.1 μM (6) which display only one third the number of vaults per cell. The GLC4 revertant has a reduction in vault number that is still over 4-fold higher than the parent line. However, this line, although now sensitive to killing by 1 μM doxorubicin, is still resistant to 0.1 μM doxorubicin and therefore is more similar to the SW1573 resistant line with regard to drug sensitivity than to the SW 1573 revertant. Interestingly the vault levels in these lines (71,000 versus 85,000) are also quite similar. This suggests that absolute vault levels may directly dictate the extent of drug resistance. Another factor which may influence the level of vault induction may be related to the tissue specific distribution of vaults and the cancer type. Thus cells with higher endogenous vault levels, might be primed to become drug resistant and can do so with a relatively modest induction of vault levels. This finding is consistent with the distribution of vaults in tissues with the highest level of exposure to xenobiotics where greater levels of endogenous vaults could predispose these tissues to drug resistance (23). Interestingly, the lowest level of vaults was found in the MCF7/S cell line (10,633 vaults/cell). This line was derived from a breast carcinoma, and its drug-resistant derivative MCF7/MR (39,000 vaults/cell), has a level of vaults comparable to the drug sensitive SW1573/S and 8226/S cell lines and might reflect a different required level of vaults to achieve drug resistance in this tissue type.

The mechanism of vault up-regulation is unknown. Although the overexpression of vaults in MDR cells is intriguing, this does not prove that vaults are responsible for drug resistance. We hypothesize that vault overexpression is a critical component of a pathway involved in non-Pgp MDR and that the mechanism of vault-mediated MDR may be through vault binding directly to drugs or possibly through vault interaction with a protein or RNA that binds drugs.

Note added in proof—A search of the GenBank™ data base revealed another potential member of the human vRNA gene family (HVGX) on chromosome X, accession number Z97054 located at bases 558392–568492.

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REFERENCES

1. Roninson, I. B. (1991) Molecular and Cellular Biology of Multidrug Resistance in Tumor Cells (Roninson, I. B., ed.), Plenum Press, New York
2. Cole, S. P., Bhunwraj, G., Gerlach, J. H., Mackie, J. E., Grae, C. E., Almquist, K. C., Stewart, A. J., Kurz, E. U., Duncan, A. M., and Deleley, R. G. (1992) Science 258, 1650–1654
3. Grant, C. E., Valdmanarsson, G., Hipffer, D. R., Almquist, K. C., Cole, S. P., and Deleley, R. G. (1994) Cancer Res. 54, 357–361
4. Zaman, G. J., Flens, M. J., van Leusden, M. R., de Haas, M., Mulder, H. S., Lankelma, J., Pinedo, H. M., Scheper, R. J., Baas, F., Broxterman, H. J., and Borst, P. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 8822–8826
5. Futscher, B. W., Abbaszadegan, M. R., Domann, F., and Dalton, W. S. (1994) Biochem. Pharmacol. 47, 1601–1606
6. Schepfer, R. J., Broxterman, H. J., Scheffer, G. L., Kooij, M., Dalton, W. S., van Heijningen, T. H., van Kalken, C. K., Slovak, M. L., de Vries, E. G., van der Valk, P., Meijer, C. J. L. M., and Pinedo, H. M. (1993) Cancer Res 53, 1475–1479
7. Izquierdo, M. A., van der Zee, A., Vermeeren, J. B., van der Valk, P., Belien, J. A. M., Giaccone, G., Scheffer, G. L., Flens, M. J., Pinedo, H. M., Kenenans, P., Meijer, C. J. L. M., de Vries, E. G. E., and Schepfer, R. J. (1995) J. Natl. Cancer Inst. 87, 1230–1235
8. List, A. F., Spierer, C. A., Grogan, T. M., Johnson, C., Roe, D. J., Greer, J. P., Wolff, S. N., Broxterman, H. J., Scheffer, G. L., Scheper, R. J., and Dalton, W. S. (1996) Blood 87, 2464–2469
9. Scheffer, G. L., Wijngaard, P. L. J., Flens, M. J., Izquierdo, M. A., Slovak, M. L., Pinedo, H. M., Meijer, C. J. L. M., Clevers, H. C., and Schepfer, R. J. (1995) Natl. Med. 1, 578–582
10. Kederhsa, N. L., and Rome, L. H. (1986) J. Cell Biol. 103, 699–709
11. Rome, L., Kederhsa, N., and Chugani, D. (1991) Trends Cell Biol. 1, 47–50
12. Kisschweier, V. A., Vass, S. R., and Rome, L. H. (1996) Trends Cell Biol. 6, 174–178
13. Kederhsa, N. L., and Rome, L. H. (1996) Anal. Biochem. 156, 161–170
14. Kederhsa, N. L., House, J. E., Chugani, D. C., and Rome, L. H. (1991) J. Cell Biol. 112, 225–235
15. Kisschweier, V. A., Searles, R. P., Kederhsa, N. L., Garber, M. E., Johnson, D. L., and Rome, L. H. (1993) J. Biol. Chem. 268, 7688–7673
16. Kederhsa, N. L., Miquel, M. C., Bittner, D., and Rome, L. H. (1990) J. Cell Biol. 110, 895–901
17. Chugani, D. C., Rome, L. H., and Kederhsa, N. L. (1993) J. Cell Sci. 106, 23–29
18. Kuiper, C. M., Broxterman, H. J., Baas, F., Schuurhuis, G. J., Huisman, H. J., Scheffer, G. L., Lankelma, J., and Pinedo, H. M. (1990) J. Cell. Biol. 110, 35–41
19. Zijlstra, J. G., de Vries, E. G. E., and Mulder, N. H. (1987) Cancer Res. 47, 1780–1784
20. Versavonvort, C. H., Withoff, S., Broxterman, H. J., Kuiper, C. M., Scheper, R. J., Mulder, N. H., and de Vries, E. G. (1995) Int. J. Cancer 61, 375–380
21. Taylor, C. W., Dalton, W. S., Parrish, P. R., Gleason, M. C., Bellamy, W. T., Thompson, P. H., Roe, D. J., and Trent, J. M. (1991) Br. J. Cancer 64, 923–929
22. Schepfer, R. J., Dalton, W. S., Grogan, T. M., Schlosser, A., Bellamy, W. T., Taylor, C. W., Scuderi, P., and Spier, C. (1993) Int. J. Cancer 52, 562–567
23. Izquierdo, M. A., Scheffer, G. L., Flens, M. J., Giaccone, G., Broxterman, H. J., Meijer, C. J., van der Valk, P., and Scheper, R. J. (1996) Am. J. Pathol. 148, 877–887