ABSTRACT

Vaults may contribute to multidrug resistance by transporting drugs away from their subcellular targets. To study the involvement of vaults in the extrusion of anthracyclines from the nucleus, we investigated the handling of daunorubicin by drug-sensitive and drug-resistant non-small lung cancer cells, including a green fluorescent protein (GFP)-tagged major vault protein (MVP)-overexpressing transfectant (SW1573/MVP-GFP). Cells were exposed to 1 μM daunorubicin for 60 min, after which the cells were allowed to efflux the accumulated drug. No significant differences in daunorubicin efflux kinetics were observed between the sensitive SW1573 and SW1573/MVP-GFP transfected, whereas the drug-resistant SW1573/2R120 cells clearly demonstrated an increased efflux rate. It was noted that the redistribution of daunorubicin from the nucleus into distinct vesicular structures in the cytoplasm was not accompanied by changes in the intracellular localization of vaults. Similar experiments were performed using mouse embryonic fibroblasts derived from wild-type and MVP knockout mice, which were previously shown to be devoid of vault particles. Both cell lines showed comparable drug efflux rates, and the intracellular distribution of daunorubicin in time was identical. Reintroduction of a human MVP tagged with GFP in the MVP−/− cells results in the formation of vault particles but did not give rise to an altered daunorubicin handling compared with MVP−/− cells expressing GFP. Our results indicate that vaults are not directly involved in the sequestration of anthracyclines in vesicles nor in their efflux from the nucleus.

INTRODUCTION

The vault complex is an evolutionarily conserved ribonucleoprotein particle that is composed of multiple copies of three high M₆ proteins and small untranslated RNA molecules of 88–141 bases (vRNAs; reviewed in Refs. 1–3). The main component is the M₆ 100,000 major vault protein (MVP), also referred to as lung resistance-related protein (LRP), which determines the structure of the vault. A M₆ 193,000 vault poly(ADP-ribose) polymerase together with the M₆ 240,000 telomerase-associated protein (TEP1) constitute the minor vault proteins (4, 5). The term “vault” describes the morphology of the particles, which contain multiple arches reminiscent of vaulted ceilings in cathedrals. The vault components are arranged into a hollow barrel-like structure measuring approximately 35 × 65 nm, with 8-fold symmetry, an invaginated waist, and two protruding caps (6–8). Mammalian vaults are predominantly localized in the cytoplasm; a small fraction (≈5%) of vaults is found associated with the nucleus (9, 10). The precise cellular function of the complex is still unknown, but there is some evidence that vaults play a role in cellular detoxification processes and consequently contribute to the multidrug resistance (MDR) phenotype frequently observed in cancer cells.

A link between vaults and MDR was first suggested when the LRP was identified as the human MVP (11). Previously, LRP had been found overexpressed in several non-P-glycoprotein (P-gp) multidrug-resistant cell lines (12). It was proposed that vaults function in MDR by mediating the extrusion of drugs from the nucleus and/or the sequestration of drugs into exocytotic vesicles. In subsequent studies, it was established that MVP/LRP (vault) expression correlated with a chemoresistant phenotype in several cancer cell lines and primary tumor samples (13–16). In addition, several—not but all—clinical studies indicated that MVP/LRP expression at diagnosis was an independent adverse prognostic factor for response to chemotherapy (reviewed in Refs. 2 and 17). Until now, few studies have attempted to directly assess the contribution of vaults to drug resistance. Stable expression of MVP in the ovarian carcinoma cell line A2780 led to increased numbers of vault particles but failed to confer drug resistance to etoposide, vindesine, and doxorubicin (11, 18). In contrast, experiments by the Akiyama group (19–21) provide support for a role of vaults in the extrusion of anthracyclines from the nucleus. Kitazono et al. (21) demonstrated that treatment of the human colorectal carcinoma SW-620 cells with sodium butyrate resulted in a strong induction of MVP and made the cells less sensitive to doxorubicin, etoposide (VP-16), vincristine, and paclitaxel. The expression of MVP-specific ribozymes reversed the observed drug-resistant phenotype. Focusing on the molecular mechanism of vault-mediated resistance against anthracyclines, they showed that doxorubicin was effluxed more rapidly from the nuclei of sodium butyrate-treated vault-overexpressing cells compared with nontreated cells. Moreover, the nuclear doxorubicin efflux could be inhibited by the addition of polyclonal anti-MVP antibodies (21). Recently, we generated an MVP knockout mouse model in which vault particles are absent (22). We tested the sensitivity of MVP-deficient cells to a panel of cytostatic agents and found that neither embryonic stem cells nor bone marrow cells showed an increased sensitivity to these drugs compared with wild-type cells. It was concluded that disruption of the murine MVP gene did not induce hypersensitivity to cytostatics.

To study how drugs are handled in the cell in the presence and absence of vaults, we examined the influx and efflux kinetics and subcellular distribution of the fluorescent anthracycline daunorubicin. We compared mouse embryonic fibroblasts (MEFs) derived from wild-type and MVP−/− mice and the drug-sensitive human non-small cell lung cancer cell line SW1573 and its drug-resistant vault-overexpressing derivative SW1573/2R120. We examined the drug-handling abilities of transfectants stably expressing a green fluorescent protein (GFP)-labeled MVP, which is incorporated into vault particles, enabling us to monitor vault distribution in the challenged cells.

MATERIALS AND METHODS

Cell Lines and Culture Conditions. The cell lines SW1573, its doxorubicin-selected multidrug-resistant variant SW1573/2R120 (23), and the transfectant SW1573/MVP-GFP (24) were maintained in DMEM (Life Technologies, Inc., Paisley, Scotland), supplemented with 10% (v/v) FCS, 1 mM pyruvate, 100 units/ml penicillin, and 100 μg/ml streptomycin (Life Technol-
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RESULTS

Efflux Rates and Intracellular Distribution of Daunorubicin in Non-Small Cell Lung Cancer Cells. The drug-sensitive non-small cell lung cancer cell line SW1573 and its drug-resistant derivative SW1573/2R120 were cultured in the presence of 1 μM anthracycline daunorubicin for 1 h, after which the drug was washed away and the cells were allowed to efflux the drug. At regular intervals, we monitored the intracellular daunorubicin fluorescence by FACScan (Fig. 1A). It is clear that the extrusion of daunorubicin from the drug-resistant SW1573/2R120 occurs far more efficiently than in the drug-sensitive parental cells, resulting in a reduction of the intracellular fluorescence after a 4-h efflux period to 20% of the fluorescence present in the cells after the influx period. By contrast, 70% of the initial fluorescence was still detected in the SW1573 cells after an efflux of 4 h. Fig. 1A shows that the initial clearance rate of daunorubicin appears to be rapid, after which it levels off, suggesting the involvement of different mechanisms. The intrinsic fluorescence of daunorubicin also enabled us to examine its intracellular distribution in time. Daunorubicin was initially directed to the nucleus in both cell lines (Fig. 1B, e and i). Interestingly, in the resistant cell line SW1573/2R120, there was a rapid shift of daunorubicin fluorescence from the nucleus to the cytoplasm, where it appeared in typical vesicular structures in the perinuclear region (Fig. 1B, f). After 40 min of drug efflux, the nuclei of SW1573/2R120 cells were almost cleared from drug, whereas daunorubicin was still clearly detectable in the nuclei of
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Expression of a GFP-Tagged MVP in SW1573 Does Not Alter the Efflux Kinetics and Intracellular Distribution of Daunorubicin. To investigate whether an overexpression of vaults is responsible for the observed drug-handling differences, the SW1573 cell line was stably transfected with a construct encoding a COOH-terminal GFP-tagged MVP (SW1573/MVP-GFP). We previously demonstrated that expression of MVP-GFP in SW1573 resulted in a 4-fold increase in MVP levels (see also Fig. 2A) and that the GFP-tagged MVP molecules were incorporated into vault particles that could be pelleted at 100,000 × g (24). To examine whether vault particles containing MVP-GFP have characteristics similar to wild-type vaults, we further fractionated the 100,000 × g pellet fraction on a 20–60% sucrose step gradient. Fig. 2B shows the results of an immunoblotting experiment in which sucrose gradient fractions were analyzed for the presence of MVP and/or MVP-GFP. Both proteins were predominantly recovered in 40–45% sucrose fractions (fractions 5–7) comparable with wild-type vaults. Furthermore, MVP has been identified as the vault subunit whose levels are limiting in the formation of vaults (16, 18), implying that the expression of MVP-GFP results in increased levels of vault particles. When challenged with daunorubicin, the behavior of the SW1573/MVP-GFP transfectant was indistinguishable from that of the drug-sensitive SW1573 cells (Fig. 1, A and B, a–d), indicating that a raised level of vaults is not sufficient to give rise to an increased daunorubicin efflux or an intracellular redistribution of the fluorescent drug, as observed in SW1573/2R120 cells. The expression of the GFP-tagged MVP allowed us to assess the effects of daunorubicin on the vault compartment. As shown in Fig. 1C, the dispersed cytoplasmic distribution of the vaults did not change during the drug treatment. Particularly, vaults were never found to accumulate near the daunorubicin-filled vesicles or at the nuclear membrane, which is what we would expect to observe if vaults were actually engaged in shuttling drug from the nucleus to cytoplasmic vesicles.

Daunorubicin Handling of MEFs Lacking the MVP. We examined the redistribution and efflux of daunorubicin in MEF cells derived from wild-type and MVP−/− littermates. Previously, it was shown that vault particles were absent in MVP knockout tissues and cells (22). Furthermore, it was reported that the absence of MVP directly affected the stability of the other vault components, making the presence of a rudimentary vault complex highly unlikely (22).
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DISCUSSION

MDR in tumor cells is a significant clinical issue because it directly affects the efficacy of chemotherapy. MDR cells are characterized by a decreased accumulation of various drugs, which can be caused by the expression of drug transporters like P-glycoprotein, MRP1, and BCRP (29). However, changes in drug accumulation cannot fully account for the anthracycline resistance in many MDR cells, and other mechanisms have to contribute to the MDR phenotype. Vaults have been associated with intracellular drug transport and drug sequestration. There is experimental evidence that vaults are directly involved in the removal of anthracyclines from the nucleus (19–21). The mechanistic model that is postulated involves vaults in the nuclear membrane and/or nuclear pore complex where they function as drug efflux pumps. To test whether vaults function in the handling of anthracyclines, we investigated drug influx and efflux kinetics and its subcellular localization in MEFs derived from wild-type and MVP knockout mice as well as drug-resistant and -sensitive human nonsmall lung cancer cells.

We studied the daunorubicin efflux from a known drug-resistant vault-overexpressing cell line SW1573/2R120 and its drug-sensitive parent SW1573. Furthermore, we included a stable transfected SW1573 cell line expressing a GFP-tagged MVP in our studies. Comparison of SW1573 with its MVP-overexpressing transfected (SW1573/MVP-GFP) revealed no changes in daunorubicin efflux rate and intracellular distribution, although vault particles were formed composed of MVP and MVP-GFP. By contrast, the resistant derivative SW1573/2R120 clearly displayed a higher drug efflux rate, resulting in a reduced drug accumulation in agreement with reports in the literature (30, 31). We observed that daunorubicin was directed to the nucleus and subsequently transferred from the nucleus to cytoplasmic vesicles. This process occurs earlier and more efficiently in the resistant cells. When we compared wild-type MEFs with MVP knockout MEFs in a similar experimental set-up, we did not detect differences in the handling of daunorubicin between these cell lines. Experiments in which we overexpressed GFP-tagged vaults in knockout MEFs gave similar results.

The sequestration of anthracyclines in cytoplasmic structures/vesicles has been described in several drug-resistant cell lines and has been associated with the expression of P-gp, MRP1, and/or MVP (32–40). However, much is still unclear about the sequestration process and its role in MDR. It is believed that the cytoplasmic structures that sequester anthracyclines are acidic in nature, like lysosomes, trans-Golgi network, and endosomes (36–38, 40, 41). Anthracyclines, being weak bases, accumulate in these acidic compartments where they are retained because they are protonated, becoming membrane-impermeable. Nevertheless, if the acidic nature of the anthracyclines is the only driving force for the sequestration in the cytoplasmic vesicles, one would expect this to occur during the influx phase as well. However, this is in contrast with our observation of the

Fig. 2. MVP and GFP-tagged MVP expression levels and the formation of GFP-tagged vault particles. A, cell lysates were prepared from SW1573, SW1573/2R120, and SW1573/MVP-GFP and from wild-type (MVP+/+) and MVP−/− MEFs as well as an MVP−/− transfectant-expressing GFP-MVP. Ten µg of total protein were subjected to SDS–PAGE. Size-fractionated proteins were transferred to nitrocellulose, after which MVP and GFP-tagged MVP were detected with anti-MVP. Note that murine MVP is somewhat smaller in size than human MVP. B, to examine whether the GFP-tagged MVP proteins expressed in the SW1573 and MVP−/− cells were incorporated into vault particles, vaults were purified from cell lysates by differential centrifugation. A 100,000 × g pellet enriched in vaults was further fractionated on a sucrose step gradient as described in “Materials and Methods.” The MVP-GFP in SW1573 as well as the GFP-MVP in MVP−/− MEFs were almost completely recovered in the 40–45% sucrose gradient fractions (fractions 5–7), similar to regular vault particles in SW1573 and MVP−/− cells. Note that different amounts of starting material were loaded on the gradients.

Fig. 3A shows the intracellular daunorubicin fluorescence of the MEFs during the daunorubicin uptake and subsequent efflux. No differences were observed between wild-type cells and the cells lacking vaults. Comparison of the daunorubicin efflux rates in the MEFs with the rates observed in non-small cell lung cancer cells (Fig. 1A) reveals that MEFs are more efficient in the removal of daunorubicin. This might in part be caused by the functional expression of several ABC transporters in MVP+/+ and MVP−/− MEFs (Table 1). P-gp and MRP1 activities were comparable in wild-type and knockout MEFs, whereas BCRP activity was found to be higher in MVP+/+ MEFs. However, this did not result in an increased daunorubicin extrusion, most likely because anthracyclines like daunorubicin are relatively poor substrates for BCRP (27). In agreement with the rapid clearance of daunorubicin is the rapid redistribution of daunorubicin from the nucleus to the vesicles in the cytoplasm (Fig. 3B). Because we did not observe a significant difference between wild-type and vault-deficient cells, we conclude that vaults do not play a role in either the extrusion of daunorubicin from the cells or the intracellular redistribution of the drug. Reintroduction of an expression construct encoding human MVP tagged at its NH₄ terminus with GFP in MVP−/− cells results in increased steady-state MVP levels and the formation of vault particles as judged from the recovery of GFP-MVP in gradient fractions at sucrose densities where regular vault particles end up (Fig. 2; see footnote 4). However, expression of the GFP-tagged MVP in these cells did not give rise to extra daunorubicin extrusion compared with MVP−/− MEFs transfected with a GFP expression construct (Fig. 3C). It was noted that vault particles containing GFP-tagged MVPs associate with the minor vault proteins vault poly(ADP-ribose) polymerase and TEP1 as well as the vRNA. In addition, GFP-tagged vaults show a similar response to a lower temperature (formation of vault tubes) as genuine vault particles, suggesting that these vaults are fully assembled and functional (24; see footnote 4).

Taken together, our results suggest that vaults are not directly involved in the sequestration of daunorubicin in cytoplasmic vesicles and/or the extrusion of drugs from the nucleus.

Fig. 3B shows the intracellular daunorubicin fluorescence of the MEFs during the daunorubicin uptake and subsequent efflux. No differences were observed between wild-type cells and the cells lacking vaults. Comparison of the daunorubicin efflux rates in the MEFs with the rates observed in non-small cell lung cancer cells (Fig. 1A) reveals that MEFs are more efficient in the removal of daunorubicin. This might in part be caused by the functional expression of several ABC transporters in MVP+/+ and MVP−/− MEFs (Table 1). P-gp and MRP1 activities were comparable in wild-type and knockout MEFs, whereas BCRP activity was found to be higher in MVP+/+ MEFs. However, this did not result in an increased daunorubicin extrusion, most likely because anthracyclines like daunorubicin are relatively poor substrates for BCRP (27). In agreement with the rapid clearance of daunorubicin is the rapid redistribution of daunorubicin from the nucleus to the vesicles in the cytoplasm (Fig. 3B). Because we did not observe a significant difference between wild-type and vault-deficient cells, we conclude that vaults do not play a role in either the extrusion of daunorubicin from the cells or the intracellular redistribution of the drug. Reintroduction of an expression construct encoding human MVP tagged at its NH₄ terminus with GFP in MVP−/− cells results in increased steady-state MVP levels and the formation of vault particles as judged from the recovery of GFP-MVP in gradient fractions at sucrose densities where regular vault particles end up (Fig. 2; see footnote 4). However, expression of the GFP-tagged MVP in these cells did not give rise to extra daunorubicin extrusion compared with MVP−/− MEFs transfected with a GFP expression construct (Fig. 3C). It was noted that vault particles containing GFP-tagged MVPs associate with the minor vault proteins vault poly(ADP-ribose) polymerase and TEP1 as well as the vRNA. In addition, GFP-tagged vaults show a similar response to a lower temperature (formation of vault tubes) as genuine vault particles, suggesting that these vaults are fully assembled and functional (24; see footnote 4).

Taken together, our results suggest that vaults are not directly involved in the sequestration of daunorubicin in cytoplasmic vesicles and/or the extrusion of drugs from the nucleus.
initial accumulation of the drug in the nucleus in all cell lines tested. Reports as to whether sequestration itself contributes to MDR are conflicting as is the evidence that the vesicles that accumulate anthracyclines are exocytic in nature (35–38, 40–44). Nevertheless, treatment of resistant cells with carboxylic ionophores (like monensin and nigericin), brefeldin A, and chloroquine, which disrupt intracellular vesicular transport and raise intracellular pH, results in increased anthracycline accumulation and redistribution of drug to the nucleus (36, 38, 41). Like our results, these observations suggest that the sequestration of drug in cytoplasmic vesicles reduces the amount of drug in the nucleus.

Several clinical studies recognize MVP/LRP expression as an independent negative prognostic marker for response on chemotherapy and/or disease-free survival or overall survival (review in Ref. 2). However, most of these studies investigated relatively small numbers of patients and used a limited array of MVP detection techniques, notably immunohistochemistry and reverse transcription-PCR. Compelling evidence that MVP expression correlates with the clinical response and prognosis is still lacking and should come from a prospective trial and a multivariate analysis of risk factors. The main question is whether vaults play a direct role in drug resistance, e.g., as drug transporter, or whether vaults are merely a marker of a drug resistance phenotype and/or general stress response. This putative function of vaults in stress response (aimed at prolonging cell survival) may also explain the observations of Kitazono et al. (21) in butyrate-treated colon carcinoma cells, which overexpress vaults and display resistance against several cytotoxic agents. Especially when vaults play an essential role in the stress response, one could envisage why the resistance phenotype is (partially) reversed upon the expression of MVP specific ribozymes; however, this does not necessarily mean that vaults are actively engaged in drug transport in these cells.

The presented data as well as our analyses of the MVP knockout mice (22) indicate that vaults do not function directly as drug transporters. However, we cannot exclude that vaults help the cell to deal with various forms of stress and as such contribute to a drug-resistant phenotype. In this respect, the main task that remains is the elucidation of the cellular function of the vault complex.

ACKNOWLEDGMENTS

We thank George Scheffer (Department of Pathology, Free University Medical Center, Amsterdam, The Netherlands) and Erna Fränzel-Luijtens (Department of Hematology, Erasmus Medical Center, The Netherlands) for helpful comments during the preparation of the manuscript.

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Efflux Kinetics and Intracellular Distribution of Daunorubicin Are Not Affected by Major Vault Protein/Lung Resistance-Related Protein (Vault) Expression

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*Cancer Res* 2004;64:4887-4892.

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