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ARTICLE *in* INTERNATIONAL JOURNAL OF CANCER · SEPTEMBER 2005

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Int. J. Cancer: **116,** 703–709 (2005)
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Probing ATP-dependent conformational changes in the multidrug resistance protein 1 (MRP1/ABCC1) in live tumor cells with a novel recombinant single-chain Fv antibody targeted to the extracellular N-terminus

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The multidrug resistance protein 1 (MRP1/ABCC1) is an ATP-driven transporter that mediates the cellular extrusion of various chemotherapeutic agents. We have previously isolated a novel recombinant single-chain Fv antibody (A5scFv), which specifically targets the extracellular N-terminus of the human MRP1 expressed on the surface of live tumor cells. Fusion of A5scFv to *Pseudomonas* exotoxin revealed an immunotoxin that bound to the immobilized MRP1-derived peptide upon ELISA, but surprisingly failed to recognize MRP1 on the surface of live tumor cells. As these results suggested that the N-terminus of MRP1 has a limited accessibility to the extracellular space, we used the A5scFv antibody to probe for putative conformational changes that might occur in viable tumor cells upon ATP binding. A5scFv recognized viable MRP1-expressing cells with intact ATP pools, whereas ATP depletion resulted in the loss of A5scFv reactivity. Consistently, restoration of cellular ATP levels resulted in resumption of A5scFv binding to MRP1 in live tumor cells. Flow cytometric analysis confirmed that ATP-depleted cells accumulated significantly higher levels of the established substrate calcein AM, whereas after restoration of cellular ATP pools, cells displayed a much lower level of calcein AM accumulation. Moreover, pretreatment of MRP1-expressing cells with the membrane fluidizer benzyl alcohol resulted in a dramatic increase in A5scFv reactivity, suggesting that membrane fluidization results in the exposure of the N-terminus of MRP1 to the extracellular milieu. These results constitute the first extracellular probing of the putative conformational changes that MRP1 adopts in viable tumor cells upon ATP binding. Furthermore, although ATP binding occurs in the cytosolic nucleotide binding domains of MRP1, significant conformational changes are apparently propagated to the N-terminus residing at the extracellular compartment.
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Several members of the large ATP-binding cassette (ABC) superfamily of transport proteins have the facility to translocate an extraordinarily diverse array of structurally dissimilar endogenous and exogenous substrates and their metabolites across cell membranes.[1,2] Among these are 3 important anticancer drug efflux transporters: the multidrug resistance protein 1 (MRP1/ABCC1),[3,4] breast cancer resistance protein (BCRP/ABCG2)[5] and P-glycoprotein (Pgp/ABCB1).[1,2,6,7] Overexpression of these MDR efflux transporters results in an ATP-dependent decrease in drug accumulation in malignant cells.[1,2,6,7] Consequently, overexpression of MRP1, BCRP, or Pgp results in the acquisition of MDR to multiple anticancer drugs. Increased expressions of MRP1, Pgp and BCRP have been documented in hematologic malignancies and solid tumors, suggesting an important role for these transporters in the potential conferring of clinical drug resistance upon malignant cells.[5–9]

Chemotherapeutic agents continue to be the most effective treatment for metastatic tumors.[7] However, the ability of malignant cells to become simultaneously resistant to multiple anticancer drugs, a trait known as MDR, remains a major impediment to the successful chemotherapy of various cancers. Hence, the ability to predict and circumvent drug resistance is very likely to improve cancer chemotherapy. In this respect, gaining a better mechanistic insight into the ATP-driven efflux activity of these MDR transporters and, above all, overcoming MDR by reversal agents and chemosensitizers is of key clinical importance. As a first step toward this end, we have recently initiated studies aimed at the isolation of small recombinant single-chain Fv (scFv) antibodies, which specifically target extracellular domains of these important MDR efflux transporters. Using a phage display approach, we have recently isolated 2 scFv antibodies that specifically bind to the first extracellular loop of Pgp in live tumor cells.[10,11] We further showed that one of these recombinant antibodies (termed A2scFV) abolished the drug efflux activity of Pgp and thereby reversed the MDR phenotype of human carcinoma cells to doxorubicin.[11] Extending on these studies, we recently isolated another recombinant scFv antibody (termed A5scFV) that specifically targets an extracellular N-terminal epitope of the human MRP1 as expressed on the surface of viable tumor cells with MRP1 overexpression.[12] In an attempt specifically to target and eradicate MDR malignant cells with MRP1 overexpression, we fused the A5scFv antibody to *Pseudomonas* exotoxin. This immunotoxin bound to the immobilized MRP1-derived peptide upon ELISA, but surprisingly failed to recognize MRP1 on the surface of live tumor cells. As these results suggested that the N-terminus of MRP1 is not easily accessible to the extracellular milieu, we used the A5scFv antibody to probe for putative conformational changes that might occur in MRP1 upon ATP binding in live tumor cells. The A5scFv antibody recognized viable MRP1-expressing cells with intact ATP pools, but completely lost reactivity upon ATP depletion. Hence, our results provide the first extracellular probing of the putative conformational changes that MRP1 adopts in viable tumor cells upon ATP binding. Furthermore, whereas ATP binding occurs in the cytosolic nucleotide binding domains (NBDs) of MRP1, conformational changes are apparently propagated to the extracellular N-terminus.

Material and methods

Cell lines

Human ovarian carcinoma 2008 cells and their 2008/MRP1 subline obtained by stable transduction of the human MRP1 cDNA were kindly provided by Professor P. Borst (Netherlands Cancer Institute, Amsterdam, The Netherlands). GLC4/Adr cells were established by doxorubicin stepwise selection of human GLC4 small cell lung cancer cells and were maintained in the presence of 1 μM doxorubicin.[13] Pyr^{R100}, a pyrimethamine-resistant subline of Chinese hamster ovary (CHO) AA8 cells,[14] which has completely lost MRP1 expression,[15] was continuously grown in the presence of 100 μM pyrimethamine and 1 mM sodium pyruvate. All cell lines were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum (Biological Industries, Beth-Haemek, Israel), 2 mM L-glutamine, penicillin and streptomycin (Gibco-BRL, Gaithersburg, MD).

Abbreviations: BCRP, breast cancer resistance protein; MDR, multidrug resistance; MRP1, multidrug resistance protein 1; NBD, nucleotide binding domain; PE, *Pseudomonas* exotoxin; Pgp, P-glycoprotein; scFv, single-chain Fv.

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Received 14 November 2004; Accepted after revision 16 February 2005
DOI 10.1002/ijc.21124
Published online 18 April 2005 in Wiley InterScience (www.interscience.wiley.com).

Generation of soluble scFv-PE38 fusion protein

For the construction of the A5scFv-PE38 immunotoxin, the scFv gene (Genebank accession number AY270182) was subcloned as an *Nde*I-*Hind*III fragment into the plasmid pULI-7, which encodes the truncated form of the translocation and ADP-ribosylation domains of *Pseudomonas* exotoxin (PE38).[16] A5scFv-PE38 was produced in *E. coli* BL21 λDE3 cells. Expression was induced by IPTG. Then, the immunotoxin was purified and refolded as previously described.[10] Properly folded scFv-PE38 was separated from contaminating proteins and aggregates of improperly folded polypeptides by sequential ion-exchange chromatography on Q-Sepharose and Mono Q.[17,18] The purity of the A5scFv-PE38 protein was estimated by SDS-PAGE on nonreducing 10% polyacrylamide gels and the suitable fractions were kept at −70°C until analysis. Binding specificity was performed using an ELISA assay. Maxisorb immunoplates were coated with biotinylated peptides (10 μg/ml) that were captured on BSA-Biotin (10 μg/ml; Sigma, St. Louis, MO), streptavidin-coated wells (10 μg/ml; Promega, Madison, WI) by an overnight incubation at 4°C. We used the MRP1-derived peptide 1: M^1ALRGFCSADGSDPLWDW,[18] which was specifically targeted by the recombinant A5scFv antibody, and several other MRP1 or Pgp-derived peptides, as described previously.[12] Following blocking with 2% skim-milk in PBS (Biological Industries, Beth-Haemek, Israel), the purified A5scFv-PE38 immunotoxin (in sequential dilutions) was added for 1 hr at room temperature. Rabbit-anti-PE antibody (kindly provided by Dr. Ira Pastan, National Cancer Institute, Bethesda, MD) was then added as a secondary antibody for 1-hr incubation at RT, following which an incubation with antirabbit-HRP antibody (at a 1:1,000 dilution; Jackson Immunochemicals, West Grove, PA) for 1 additional hr at RT was performed. The binding of mouse serum was detected by 100 μl of TMB solution (Dako, Glostrup, Denmark) and the reaction was stopped by the addition of H_2SO_4 to a final concentration of 2 N. The absorbance was measured at a wavelength of 450 nm.

Functional assays for MRP1 efflux activity

Exponentially growing cells (1×10^6) were washed twice with PBS and incubated in PBS containing 100 nM calcein AM (Molecular Probes, Eugene, OR), a chromophore that, in its intracellular anionic form (*i.e.*, calcein), is an MRP1 substrate. After incubation for 15 min at 37°C, cells were harvested by centrifugation (4°C; 3 min; 10,000*g*), washed once with cold PBS and analyzed for fluorescence intensity using an FACS-Calibur flow cytometer. Excitation and emission were at 488 and 525 nm, respectively.

In ATP depletion and restoration experiments, washed cells were preincubated with ATP-depleting agents, *i.e.*, a combination of 1 mM potassium cyanide and 10 mM 2-deoxy-D-glucose or 1 mM sodium azide and 5 μM oligomycin (all from Sigma) for 30 min at 37°C.[19–21] For ATP restoration, these cells were first washed twice with excess PBS, then incubated for 30 min at 37°C with the ATP restoring agents sodium pyruvate (1 mM; Gibco-BRL) and D-glucose (5 mM; Sigma). These cells were examined for intracellular calcein accumulation as described above.

Flow cytometric analysis

Monolayer cells were detached from the cell culture Petri dishes after 2 washes with PBS followed by 5 min incubation at 37°C in a PBS containing 10 mM EDTA (Sigma). All following incubations and washing steps were performed in ice-cold PBS containing 0.1% BSA. Cells (10^6) were washed twice, followed by incubation with soluble purified A5scFv antibody (50 μg/ml) for 60 min on ice. The expression, purification and quantification of soluble A5scFv antibody were performed as described previously.[12] Immunofluorescence detection was achieved through FITC-labeled anti-Myc antibodies (1:100; Santa Cruz Biotechnology, Santa Cruz, CA); this Myc-peptide tag was located at the the C-terminus of the A5scFv antibody as previously described.[12]

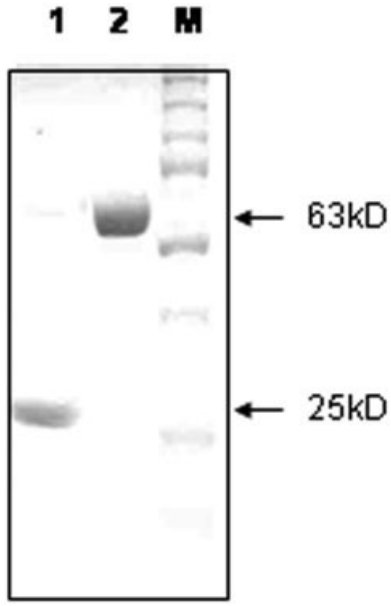

Figure 1 – SDS-PAGE analysis of purified soluble A5scFv antibody and A5scFv-PE38 immunotoxin. Transformed *E. coli* BL21 (λDE3) cells expressing the A5scFv antibody were harvested and the A5scFv antibody was purified from bacterial periplasms by metal-affinity chromatography utilizing a hexahistidine tag fused to the C-terminus of the A5scFv gene.[12] For construction of the A5scFv-PE38 fusion protein, the A5scFv gene was subcloned as an *Nde*I-*Hind*III fragment into the plasmid pULI-7, which encodes the truncated form of PE (PE38). *E. coli* BL21 cultures harboring this A5scFv-PE38 expression plasmid were induced with IPTG and harvested, and inclusion bodies were isolated as the insoluble protein fraction. Inclusion bodies were denatured, solubilized and reduced, followed by refolding in a redox-shuffling renaturation buffer. After renaturation, properly folded A5scFv-PE38 immunotoxin molecules were purified by ion-exchange chromatography on Q-Sepharose and MonoQ columns. The purity of the A5scFv (lane 1) and the A5scFv-PE38 (lane 2) proteins was examined by nonreducing 10% polyacrylamide gels containing SDS.

Flow cytometry with the A5scFv-PE38 immunotoxin was performed as described above except that the fusion protein was used as the primary antibody in a concentration of 200 μg/ml. Immunofluorescence detection was achieved using rabbit-anti-PE as a secondary antibody and antirabbit-FITC (1:100; Jackson) as the third antibody.

In order to determine the effect of the well-established membrane fluidizer benzyl alcohol,[22] cells were preincubated for 15 min with 20 mM benzyl alcohol (Sigma) that was first dissolved in 1% DMSO in PBS at 37°C. The A5scFv antibody (50 μg/ml) was added, without any prior washing steps, for incubation and detection as described above.

In order to determine the impact of intracellular ATP status on the binding of the A5scFv antibody to MRP1 on live cells, cells were preincubated with ATP-depletion agents or with agents that restore ATP pools (as described above), and then incubated with the A5scFv antibody, followed by flow cytometric analysis.

Results

Construction, production and purification of A5scFv antibody and its A5scFv-PE38 immunotoxin

We have recently reported on the isolation of a novel scFv antibody directed to a peptide that is part of the extracellular N-terminus of the human MRP1.[12] This A5scFv antibody was isolated from an immune phage display scFv library in which V genes were isolated

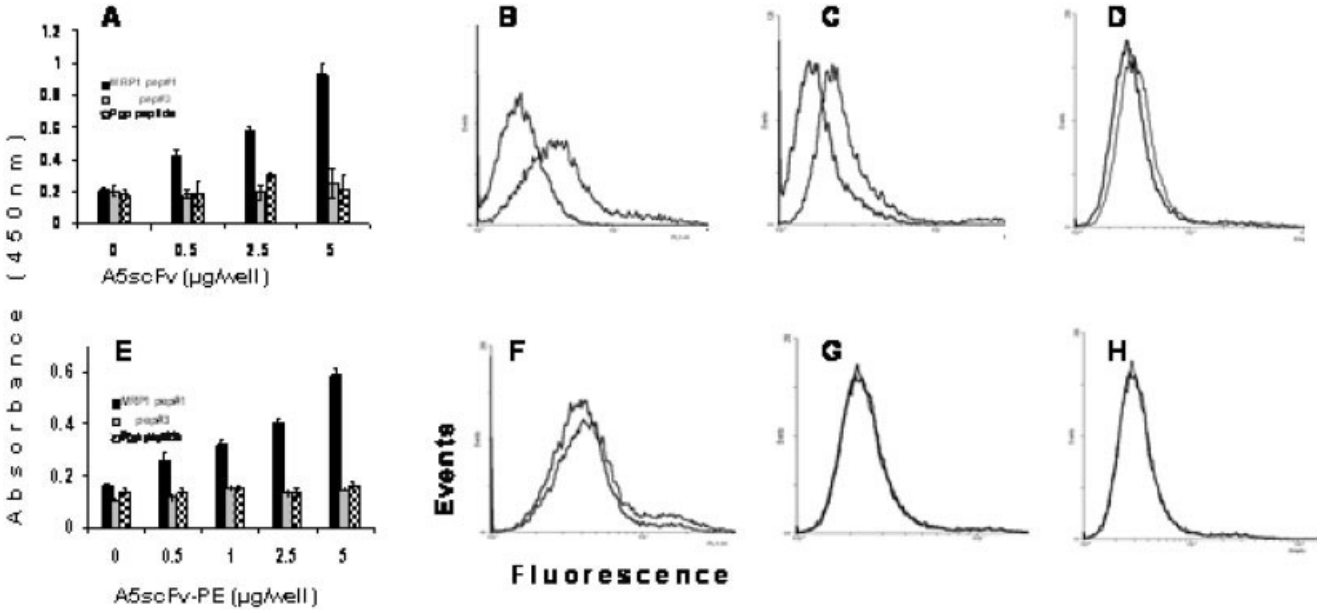

FIGURE 2 – Examination of the specificity and the binding properties of the A5scFv antibody and its A5scFv-PE38 immunotoxin. The reactivity of the A5scFv antibody (*a*) and the A5scFv-PE38 immunotoxin (*e*) with the MRP1-derived peptide 1 as well as with other MRP1 and Pgp-derived peptides was determined by ELISA. Biotynilated MRP1 and Pgp-derived peptides were immobilized to microtiter plates through a BSA-streptavidin spacer. Detection was with an HRP-conjugated anti-Myc antibody or rabbit anti-PE followed by incubation with an HRP-labeled antirabbit antibody, respectively. Color development was performed with TMB solution in 2 N H_2SO_4. The absorbance was measured at $A_{450\ nm}$. Errors bars indicate standard deviation of triplicates performed on 3 independent experiments. In order to assess A5scFv-PE binding to viable tumor cells with MRP1 overexpression, flow cytometric analysis was performed. Exponentially growing (10^6), MRP1 overexpressing GLC4/Adr cells (*b* and *f*), 2008/MRP1 cells (*c* and *g*) and MRP1-null Pyr^{R100} cells (*d* and *h*) were incubated with purified A5scFv antibody (*b–d*) or A5scFv-PE38 (*f–h*; 50 µg/ml or 200 µg/ml, respectively). The reactivity was determined with a secondary anti-Myc-FITC antibody or with rabbit-anti-PE followed by incubation with antirabbit-FITC, respectively.

from spleens of mice that were immunized with this N-terminal peptide of the human MRP1. Upon flow cytometry, the soluble A5scFv antibody revealed a high specificity toward this peptide in ELISA and it bound to viable MRP1-expressing cancer cells but failed to react with MRP1-null cells.[12] The A5scFv gene was used to construct an immunotoxin in which a truncated form of *Pseudomonas* exotoxin (PE), termed PE38, was fused to the C-terminus of the former. Subcloning, expression and purification of the immunotoxin were performed as previously described.[17,18] Highly purified A5scFv and its A5scFv-PE immunotoxin with expected sizes of 25 and 63 kDa, respectively, proved to be highly pure preparations as judged from SDS-PAGE under nonreducing conditions after purification with Mono Q chromatography (Fig. 1).

Specificity and binding properties of A5scFv and A5scFv-PE38

These pure fractions of A5scFv and A5scFv-PE38 immunotoxin were therefore analyzed by ELISA for their binding specificity to the following biotinylated peptides: the N-terminal MRP1-derived peptide (termed peptide 1) that was originally used to isolate the A5scFV antibody, a C-terminal MRP1 peptide 3 corresponding to the third extracellular loop in MSD3, as well as a Pgp-derived peptide from the first extracellular loop (termed Pgp peptide).[12] This ELISA revealed that the A5scFv antibody (Fig. 2*a*) and its A5scFv-PE38 immunotoxin reacted in a specific and dose-dependent manner with the MRP1-derived peptide 1 that was immobilized onto 96-well plates but not with the other MRP1 and Pgp peptides (Fig. 2*e*). We next assessed the ability of the A5scFv antibody and its A5scFv-PE38 immunotoxin to recognize MRP1 that is overexpressed on the surface of viable tumor cells. Flow cytometric analysis revealed that the A5scFv antibody identified MRP1 overexpressed in both GLC-4/Adr small cell lung cancer cells (Fig. 2*b*) and 2008/MRP1 ovarian carcinoma cells (Fig. 2*c*). Surprisingly, however, the A5scFv-PE38 immunotoxin failed to bind MRP1 on the surface of these viable tumor cells (Fig. 2*f* and *g*). To the best of our knowledge, the finding that an scFv antibody retains binding capacity to a cell surface antigen, whereas its scFv-PE38 fusion protein does not, has not been reported before. As expected, neither the A5scFv antibody nor its scFv-PE38 immunotoxin could react with MRP1 null cells (Fig. 2*d* and *h*, respectively).

Probing ATP-dependent conformational changes of N-terminus of MRP1 using A5scFv antibody

The above results supported the hypothesis that the N-terminal epitope of MRP1 has a limited accessibility to the extracellular space; the A5scFv fragment that is rather small in size can readily bind this epitope, however, when fused to a larger protein like the A5scFv-PE38, the binding capacity is lost. These results led us to explore the applicability of the A5scFv antibody as a molecular tool that probes for the extracellular conformational transitions that MRP1 may undergo during ATP binding and/or substrate translocation. Since ATP binding is a prerequisite for a successful transport cycle in all ABC transporters,[1,5–8] we first explored in the current article the role of ATP status on A5scFv reactivity toward MRP1. It is well established that ABC transporters including MRP1, BCRP and Pgp are ATP-dependent efflux pumps.[1,5–8] We therefore assumed that some conformational changes of MRP1 that might occur during ATP binding, ATPase activity and/or substrate translocation may alter the extracellular binding of the A5scFv antibody. Toward this end, we first used ATP-proficient and ATP-deficient cells in order to follow the binding of A5scFv to MRP1 in live tumor cells with MRP1 overexpression. To achieve ATP depletion in these tumor cells, we used the combination of potassium cyanide and 2-deoxy-D-glucose as well as oligomycin and sodium azide; treatment with these metabolic energy inhibitors including potassium cyanide and oligomycin

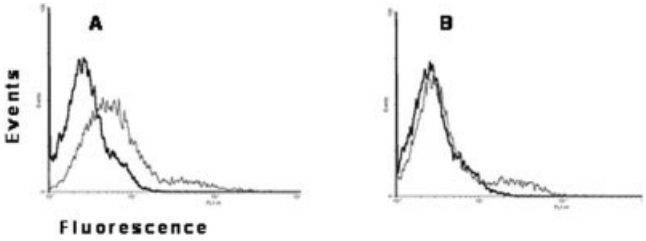

FIGURE 3 – Effect of ATP depletion on A5scFv antibody reactivity with viable MRP1 overexpressing tumor cells. Viable GLC4/Adr cells (10^6/ml) were preincubated in drug-free PBS (*a*), whereas in order to deplete intracellular ATP pools, cells were incubated for 30 min at 37°C in the presence of the metabolic energy inhibitors potassium cyanide (1 mM) and 2-deoxy-D-glucose (10 mM; *b*). Then cells were incubated with the A5scFv antibody (50 μg/ml), following which the second FITC-conjugated anti-Myc antibody was added. Live cells were then analyzed for mean fluorescence per cell using flow cytometry.

was previously shown to result rapidly in a 83–100% decrease in the intracellular ATP levels.[20,21] Using flow cytometry with viable tumor cells, we found that MRP1 binding by A5scFv was detected only prior to ATP depletion, *i.e.*, when the level of intracellular ATP was normal as in exponentially growing untreated cells (Fig. 3*a*). In contrast, following ATP depletion with potassium cyanide and 2-deoxyglucose, the binding of A5scFv to MRP1 in viable tumor cells was lost (Fig. 3*b*). Since ATP binding to the NBDs of MRP1 is an essential step in the catalytic transport cycle, the presence or absence of ATP might influence the conformational state(s) of the transporter. In order to provide further support to the ATP-dependent conformational changes in MRP1 during ATP binding and/or substrate translocation, we examined whether energy restoration would also result in resumption of A5scFv binding to MRP1-overexpressing cells. ATP depletion with a combination of potassium cyanide and 2-deoxyglucose or oligomycin and sodium azide resulted in the loss of A5scFv binding to MRP1-overexpressing cells (compare Fig. 4*b* and *c* with *a*). ATP-depleted cells were then washed, following which D-glucose and sodium pyruvate were added in order to restore intracellular ATP pools. This resulted in a complete restoration of A5scFv reactivity with MRP1-overexpressing cells (Fig. 4*d* and *e*). These findings suggest that A5scFv binding is dependent on a conformational state of the N-terminus of MRP1 only when the latter is in association with ATP.

Effect of ATP depletion on MRP1-dependent calcein AM efflux

In order to confirm the effect of ATP depletion on the functional (*i.e.*, transport) capabilities of MRP1, we used the calcein AM efflux assay on flow cytometry. Calcein is an established efflux substrate of MRP1.[19] In tumor cells with MRP1 overexpression, calcein is efficiently extruded by MRP1 in an ATP-dependent manner, thereby resulting in decreased intracellular calcein accumulation. In contrast, upon ATP depletion, MRP1 is devoid of ATP and therefore calcein cannot be extruded out of MRP1-overexpressing cells. This results in increased intracellular accumulation of this chromophore. Consistently, flow cytometric analysis after ATP depletion revealed that calcein accumulation was increased in MRP1-overexpressing cells when compared to control cells that were not ATP-depleted (Fig. 5*a*). Furthermore, restoration of ATP pools after incubation with D-glucose and sodium pyruvate resulted in a consistent decrease in intracellular calcein fluorescence with a fluorescence profile that was superimposable to control cells that were not ATP-depleted (Fig. 5*a*). The treatment of MRP1-negative cells with ATP-depleting agents did not affect the accumulation of calcein AM (Fig. 5*b*). The A5scFv molecule did not influence the function of MRP1. Incubation of MRP1 expressing cells with the scFv antibody did not have any effect on the influx and accumulation of calcein AM in these cells.

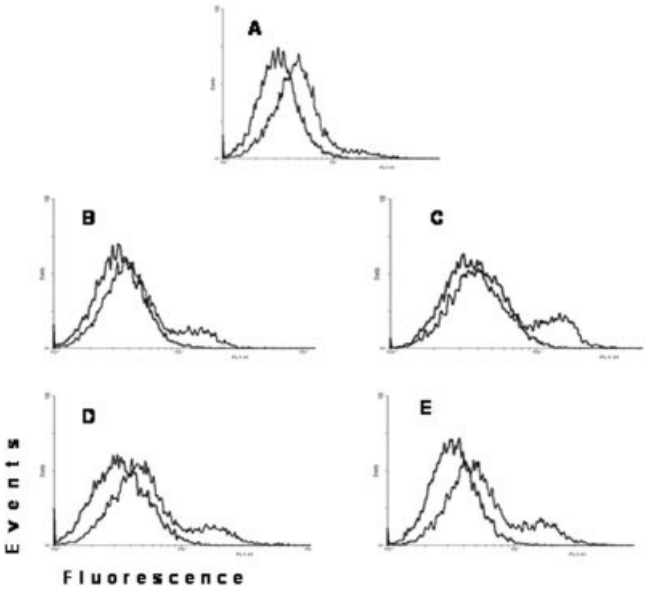

FIGURE 4 – Effect of ATP restoration on A5scFv reactivity viable MRP1 overexpressing tumor cells. Control viable 2008/MRP1 cells (10^6/ml) were preincubated in drug-free PBS (*a*), whereas in order to deplete intracellular ATP pools, cells were incubated with 1 mM potassium cyanide and 10 mM 2-deoxy-D-glucose (*b*), or with a combination of 1 mM sodium azide and 5 μM oligomycin (*c*).[20,21] Then, in order to restore intracellular ATP pools, half of the cells from each ATP-depletion treatment (*d* and *e*, respectively) were washed with PBS and the cells were coincubated with a combination of 5 mM D-glucose and 1 mM pyruvic acid. All treatments were performed for 30 min at 37°C, following which cellular fluorescence due to A5scFv binding was determined by flow cytometry.

Effect of membrane fluidizers on accessibility of MRP1 N-terminus for A5scfv binding

In order to explore the accessibility of the N-terminal epitope of MRP1 specifically, we examined the reactivity of the A5scFv antibody toward MRP1 overexpressing cells that were pretreated with benzyl alcohol, an agent that was previously reported to increase the fluidity of biologic membranes.[22] Thus, we assumed that benzyl alcohol might increase the accessibility of the N-terminus of MRP1 to the extracellular milieu via increased membrane and transporter fluidity. Indeed, whereas the A5scFv antibody reacted with GLC-4/Adr cells with MRP1 overexpression (Fig. 6*a*) but not with MRP1 null Pyr^{R100} CHO cells (Fig. 6*c*), pretreatment of GLC-4/Adr cells with benzyl alcohol resulted in a marked increase in A5scFv reactivity (Fig. 6*b*). These results support our hypothesis that the N-terminal epitope of MRP1 is indeed not easily accessible for antibody binding in its native state. The binding of the A5scFv antibody was not influenced by temperature. Cells incubated with the scFv antibody at 4°C, 20°C and 37°C exhibited similar binding pattern with the antibody (data not shown). In addition, the treatment of MRP1-expressing cells with benzyl alcohol did not influence the binding of the A5scFv-PE38 fusion protein.

Discussion

There is a growing body of literature to suggest that the N-terminus of the hMRP1 is likely to be located at the extracellular compartment. This is inferred from the following line of evidence: exploration of the sites and status of transporter N-glycosylation,[23] construction and evaluation of glycosylation-site mutants,[24] epitope insertion using small tags including hemagglutinin[25] and FLAG[26] combined with immunofluorescence, as well as detection of an extracellular N-terminal epitope in MSD1 of the hMRP1 in viable cells with transporter overexpression using novel

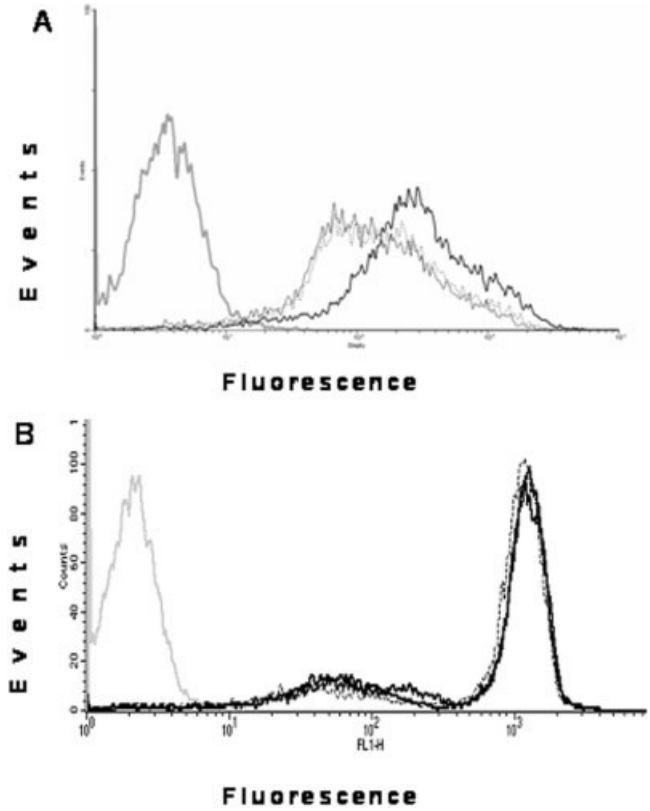

FIGURE 5 – Effect of ATP depletion and restoration on calcein AM efflux in MRP1 overexpressing cells. (*a*) Exponentially growing 2008/MRP1 cells (10^6/ml) were preincubated in drug-free PBS (thin black line), whereas another portion of cells was incubated with a combination of potassium cyanide (1 mM) and 2-deoxy-D-glucose (10 mM) in order to deplete intracllular ATP levels (thick black line). Then, in order to restore cellular ATP pools, a portion of the metabolic inhibitor-treated cells was then incubated with a combination of D-glucose and pyruvic acid (dashed line). All treatments were undertaken for 30 min at 37°C. Cells were then incubated with 100 nM calcein-AM for 30 additional min at 37°C. Then cells were transferred to ice-cold water and cellular calcein fluorescence was determined by flow cytometry. Excitation and emission were at 488 and 525 nm, respectively. The autofluorescence level of unstained (*i.e.*, calcein AM-free) cells was also recorded (thick gray line). (*b*) Wild-type GLC4 cells that do not express MRP1 were treated as above and the effect on calcein-AM accumulation was determined.

recombinant single-chain Fv antibodies.[12] While all these studies suggest that the N-terminus of the human MRP1 is extracellular, not even a single antibody has been isolated that could recognize the extracellular N-terminus of the human MRP1 or any other external epitope of this transporter. However, a monoclonal antibody (IU2H10) has been isolated that is specific to an N-terminal peptide of the hMRP1 as revealed by ELISA, Western blots, immunoprecipitation and indirect immunofluorescence.[27] Surprisingly, however, this antibody failed to react with MRP1 expressed on the surface of viable cells unless cells were permeabilized with agents such as saponin. The authors therefore concluded that the N-terminus of the hMRP1 is not accessible to this monoclonal antibody and further suggested that after exposure to the lumen of the endoplasmic reticulum, part of the N-terminus of the hMRP1 is possibly retracted back to the cytoplasm. Hence, whereas various topology experiments strongly suggest that the N-terminus of the human MRP1 is extracellular, some data suggest that at least part of this N-terminus is not accessible to the extracellular space such that monoclonal antibodies fail to react with the hMRP1 as expressed on live tumor cells. Our present study offers the first

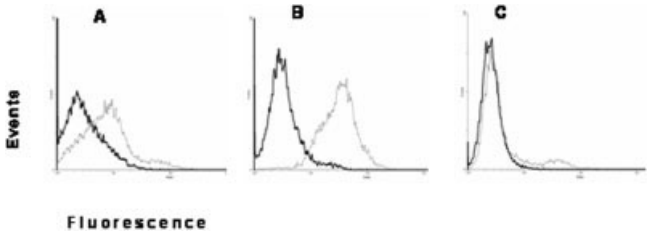

FIGURE 6 – Effect of membrane fluidizers on A5scFv antibody reactivity toward viable MRP1 overexpressing tumor cells. GLC4/Adr cells were preincubated with drug-free PBS (*a*) or with 20 mM benzyl alcohol (*b*) at 37°C for 15 min, followed by incubation with A5scFv antibody (50 μg/ml). The effects of the membrane fluidizer benzyl alcohol on A5scFv binding to MRP1 expressing cells were determined by flow cytometry. The lack of reactivity of A5scFv antibody with live MRP1-null $Pyr^{R\,r00}$ cells is also shown (*c*).

molecular reconciliation for this discrepancy. The IU2H10 monoclonal antibody was isolated after immunization of mice with the N-terminal human MRP1 peptide: M^1ALRGFCSADGSDPLWDWNVTWNTSNPDFTKCF,[33] corresponding to the first 33 amino acids of the hMRP1.[27] The epitope was subsequently mapped to 10 amino acids: S^8ADGSDPLWD.[17] In our previous study,[12] we independently used the peptide M^1ALRGFCSADGSDPLWDW,[18] which contains the S^8ADGSDPLWD[17] peptide in order to isolate our recombinant A5scFv antibody; the A5scFv antibody can efficiently bind hMRP1 in live tumor cells with transporter overexpression.[12] Hence, whereas the intact IU2H10 monoclonal antibody (IgG) fails to recognize this N-terminal hMRP epitope, our small recombinant A5scFv antibody fragment binds to this epitope in various live malignant cells with hMRP1 overexpression. These unique characteristics may have potentially important implications for the extracellular targeting of malignant MDR cells with MRP1 overexpression. Furthermore, since the A5scFv antibody is highly specific for MRP1,[12] antibodies such as A5scFv could be developed as tumor imaging agents as well as for radioimmunotherapy.[28–31] Taken altogether, it appears that at least part of the N-terminus of the hMRP1 is not easily accessible to the extracellular space and may be intimately interacting with the lipid bilayer and/or with transmembrane helices of MSD1. As previously shown, upon permeabilization with saponin, even the large IU2H10 monoclonal antibody was capable of interacting with the N-terminal epitope of the hMRP1. Clearly, further biophysical studies are necessary in order to gain more insight into the folding and accessibility of this extracellular N-terminus of the hMRP1.

In the present study, we found that the novel A5scFv antibody that we have isolated recently was able to target specifically an extracellular N-terminal epitope of the hMRP1 only when intracellular ATP pools were normal.[12] In contrast, depletion of intracellular ATP upon short-term treatment with various metabolic inhibitors resulted in the complete loss of A5scFv binding. Based on these results, we here propose that, upon ATP binding to the intracellular NBDs, the hMRP1 presumably adopts conformational changes that are propagated to its N-terminus, thereby rendering the latter more accessible to the extracellular milieu. This increased accessibility to the extracellular aqueous phase enabled its specific interaction with the small recombinant A5scFv antibody fragment, but not with the intact IU2H10 monoclonal antibody (IgG). These results are further supported by our findings described here, in which the A5scFv-PE38 fusion molecule failed to bind MRP1 on viable cells while its reactivity with the immobilized MRP1 peptide was intact (Fig. 2). These observation further indicates that the accessibility of the MRP1 N-terminal epitope is limited only to small molecules such as an scFv antibody fragment and once a larger molecule is being used, the reactivity with this epitope is lost. This proposal is consistent with the following recent reports that have studied various aspects of the conforma-

tional changes that presumably occur in the hMRP1 upon ATP binding, ATP hydrolysis and substrate translocation. One, using infrared spectroscopy with a purified hMRP1 that was subsequently reconstituted in lipid vesicles, it was found that upon MgATP binding, this transporter underwent substantial tertiary structure changes that resulted in increased accessibility of MRP1 to the solvent.[32] Remarkably, release of Pi after ATP hydrolysis caused a decrease in the accessibility of MRP1 to the water phase, thereby bringing the protein back to its initial conformation. Two, by using hydrogen/deuterium exchange kinetics and limited trypsin digestion with the above inverted proteoliposomes, it was found that upon ATP binding, hMRP1 adopts a conformation characterized by a decreased stability and/or increased accessibility to the aqueous solvent.[33] Furthermore, GSH binding induced a conformational change that increased the binding of ATP and its hydrolysis. These results therefore suggested that GSH-mediated conformational changes are essential for the coupling between drug transport and ATP hydrolysis. Three, using a different experimental approach, the vanadate-induced photocleavage reaction was employed in order to characterize the interactions of MRP1 with nucleotides and transported substrates.[34] This revealed 2 allosteric effects during the ATPase cycle of the hMRP1; ATP binding to one of the NBDs was sufficient to induce a positive allosteric effect on the formation of a prehydrolytic intermediate complex in the other site, while interaction with the transported substrates stimulated the formation of the posthydrolytic intermediates. Four, Muller *et al*.[26] employed FLAG epitope insertion into the third extracellular loop in MSD2 of the hMRP1. Using a monoclonal antibody toward the FLAG tag, they failed to detect this epitope on live MRP1-expressing cells even after deglycosylation. The authors therefore concluded that the MSD2 region, including the putative third extracellular loop, is deeply buried in the plasma membrane. Taken altogether, these findings further support the conclusion that several domains of the hMRP1 that were regarded as extracellular regions may be affected by various factors, including ATP, GSH and transported substrates in their accessibility to the extracellular aqueous solution. Our results provide the first direct evidence with live cells that the N-terminus of the hMRP1 is accessible to the extracellular space only when intracellular ATP levels are intact.

It is well established that apart from MRP1, ABC transporters including Pgp undergo conformational changes upon binding of transport substrates, MDR modulators, or after treatment with metabolic inhibitors that result in ATP-depleting agents, or when both NBDs are inactivated by mutations.[20] Thus, a monoclonal antibody (UIC2) was isolated that recognizes an extracellular N-terminal epitope of Pgp in live tumor cells.[35] Remarkably, UIC2 reactivity with Pgp expressed on the surface of intact MDR cells was increased by ATP-depleting agents as well as by the addition of various Pgp-transported substrates. Furthermore, mutational inactivation of both NBDs of Pgp also resulted in an increased reactivity of this monoclonal antibody.[20] The authors therefore concluded that Pgp adopts different conformational changes upon ATP binding, ATP hydrolysis and substrate binding. UIC2, which is a conformational antibody, traps the Pgp in a transient conformation, thereby abolishing its transport capability.[35,36] It is interesting to note that ATP depletion increases the reactivity of the UIC2 toward Pgp in live tumor cells, whereas the reactivity of our A5scFv antibody is decreased under such conditions of ATP depletion. This may possibly be due to the fact that Pgp presumably recognizes hydrophobic substrates from within the lipid bilayer,[37] whereas MRP1 functions as a classic ABC transporter that binds hydrophilic substrates in the cytosolic aqueous solution and translocates them outside the cell. This disparity between Pgp and MRP1 may translate into substantially different conformational changes in these 2 transporters. In any event, it has been recently proposed that ATP binding leads to significant conformational changes that result in dimerization of the NBDs in ABC transporters, a key step for the coupling of ATP hydrolysis to substrate translocation.[38] It would be of great clinical importance to screen our scFv library further for clones that specifically bind to MRP1 and also abolish its drug efflux activity, thereby achieving reversal of MDR.

We employed here an additional strategy to explore the accessibility of the N-terminus of MRP1 by using the membrane fluidizer benzyl alcohol and determined the binding of the A5scFv to MRP1 in live cells. Benzyl alcohol is an aromatic anesthetic that increases the fluidity of artificial membranes and biomembranes.[22] By doing so, benzyl alcohol is likely to increase the fluidity of MRP1, thereby resulting in an increased accessibility of the N-terminus of MRP1 toward the extracellular milieu. Indeed, when these agents were used in conjunction with the A5scFv, we were able to demonstrate a significant increase in A5scFv reactivity with the N-terminal MRP1 epitope. This finding is consistent with our previous results that benzyl alcohol and various nonaromatic fluidizers, including choloroform and diethyl ether, abolish the ATPase activity of Pgp, thereby resulting in loss of drug efflux activity and consequent reversal of MDR.[39] It therefore appears that, in the presence of membrane fluidizers such as benzyl alcohol, MRP1 adopts multiple conformational changes, some of which may be propagated to the extracellular N-terminus, thereby allowing for the MRP1 recognition by the A5scFv antibody.

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