Accelerated Publications

The P-Glycoprotein (ABCB1) Linker Domain Encodes High-Affinity Binding Sequences to α- and β-Tubulins†

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ABSTRACT: P-Glycoprotein (or ABCB1) has been shown to cause multidrug resistance in tumor cell lines selected with lipophilic anticancer drugs. ABCB1 encodes a duplicated molecule with two hydrophobic and hydrophilic domains linked by a highly charged region of ∼90 amino acids, the "linker domain" with as yet unknown function(s). In this report, we demonstrate a role for this domain in binding to other cellular proteins. Using overlapping hexapeptides that encode the entire amino acid sequence of the linker domain of human ABCB1, we show a direct and specific binding between sequences in the linker domain and several intracellular proteins. Three different polypeptide sequences [^{617}EKGIYFKLVTM^{627} (LDS$^{617-627}$), ^{657}SRSSLIRKRSTRRSVRGSQA^{676} (LDS$^{657-676}$), and ^{693}PVSFWRIMKLNLT^{705} (LDS$^{693-705}$)] in the linker domain interacted tightly with several proteins with apparent molecular masses of ∼80, 57, and 30 kDa. Interestingly, only the 57 kDa protein (or P57) interacted with all three different sequences of the linker domain. Purification and partial N-terminal amino acid sequencing of P57 showed that it encodes the N-terminal amino acids of α- and β-tubulins. The identity of the P57 interacting protein as tubulins was further confirmed by Western blotting using monoclonal antibodies to α- and β-tubulin. Taken together, the results of this study provide the first evidence for ABCB1 protein interaction mediated by sequences in the linker domain. These findings are likely to provide further insight into the functions of ABCB1 in normal and drug resistant tumor cells.

The successful treatment of cancer patients with chemotherapeutic drugs is often limited by the development of drug resistant tumors. Tumor cell lines selected, *in vitro*, with a single anticancer drug become resistant to a broad spectrum of chemotherapeutic drugs, hence multidrug resistant (or MDR)[1] tumor cells (for reviews, see refs *1*−*3*). The MDR phenotype of tumor cells has been associated with the overexpression of several ATP binding cassette transporters (or ABC proteins) such as the P-glycoprotein (or ABCB1), the multidrug resistance-associated protein (MRP1 or ABCC1), and the breast resistant protein (or ABCG2; for reviews, see refs *1*−*3*). These ABC transporters are members of a large family of energy-dependent transporters, consisting of 48 ABC proteins (*4*). ABCB1 is among the most-studied transporter, but its tertiary structure remains a matter of speculation (*5*). On the basis of its primary amino acid sequence, ABCB1 is tandemly duplicated with six transmembrane helices and a large cytoplasmic domain encoding an ATP binding sequence (*6*). The two halves of ABCB1 are linked by a stretch of ∼90 residues rich in polar or charged amino acids, termed the linker domain.

In normal tissues, the distribution of ABCB1 is restricted mainly to tissues with secretory functions (*7*). Its polarized localization to apical surfaces facing a lumen in the adrenal gland, liver, kidney, and intestine suggests a normal transport or detoxification mechanism. Moreover, hematopoietic stem cells and specific lymphocyte subclasses also express high levels of the protein (*8*). Although ABCB1 has been implicated in the transport of numerous ligands, the normal substrate of ABCB1 remains unknown. High levels of ABCB1 have been found in many intrinsically drug resistant tumors from colon, kidney, breast, and adrenals as well as in other tumors which had acquired the MDR phenotype after chemotherapy (for example, in acute non-lymphoblastic leukemia) (*9*, *10*). Moreover, several studies have now established an inverse correlation of ABCB1 expression and response to chemotherapy (*11*, *12*). Chan et al. (*13*, *14*) have shown that ABCB1 expression was prognostic of MDR and durable response in childhood leukemia, soft tissue sarcomas, and neuroblastomas of children. In light of these studies, there appears to be convincing evidence, at least in some cancers, that ABCB1 levels predict response to chemotherapeutic treatment (*15*).

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[1] Abbreviations: MDR, multidrug resistance; P-gp, P-glycoprotein; MRP, multidrug resistance protein; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; ABC, ATP binding cassette; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate.

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Earlier studies have shown ABCB1 to be a substrate for protein kinases C and A (*16*, *17*). Furthermore, drugs affecting protein kinase C activity modulate ABCB1 phosphorylation and MDR-mediated phenotype (*18*, *19*). In one study (*20*), PMA probol ester was shown to increase ABCB1-mediated MDR and drug efflux, while in another study (*21*), sodium butyrate treatment of SW620 human colonic carcinoma cells was shown to cause an increase in ABCB1 expression without a concomitant increase in drug resistance or efflux. Interestingly, ABCB1 in SW620 cells was shown to be poorly phosphorylated following sodium butyrate treatment (*21*). Taken together, the lack of transport function of ABCB1 in SW620 cells was not clear; however, mutations of ABCB1 phosphorylation sites within the linker domain were shown not to affect its drug transport function (*22*). By contrast, protein kinase C modulation of serine/threonine residues in the linker domain regulated the activity of an endogenous chloride channel and thus suggests that ABCB1 is a channel regulator (*23*). Thus, although it remains unclear what functions the linker domain of ABCB1 mediates, it was of interest to identify the proteins that interact with the linker domain using an *in vitro* assay. In this study, we used a large set of overlapping peptides that encode the entire primary sequence of the ABCB1 linker domain in a pulldown assay to identify ABCB1-interacting proteins from total cytosolic proteins. Our results show short sequences in the linker domain that interact with proteins with apparent molecular masses of ~80, 57, and 27 kDa. Purification and partial N-terminal amino acid sequencing of the 57 kDa protein (or P57) were revealed as α- and β-tubulins.

EXPERIMENTAL PROCEDURES

Metabolic Labeling and Cell Extraction. Drug resistant (CEM/VLB$^{1.0}$) cells were cultured in α-MEM medium supplemented with 10% fetal calf serum (Hyclon, Inc.) as previously described (*24*). All cells were examined for Mycoplasma contamination every 3 months using the Mycoplasma PCR kit from Stratagene Inc. (San Diego, CA). For metabolic labeling of cells, CEM/VLB$^{1.0}$ cells at 70−80% confluency were metabolically labeled with [^{35}S]methionine (100 μCi/mL, 1000 Ci/mmol; Amersham Life Sciences, Inc.) for 6 h at 37 °C in methionine-free α-MEM medium. Cells were then washed three times with phosphate-buffered saline (PBS), resuspended in hypotonic buffer [10 mM KCl, 1.5 mM $MgCl_2$, and 10 mM Tris-HCl (pH 7.4)] containing protease inhibitors (2 mM PMSF, 3 μg/mL leupeptin, 4 μg/mL pepstatin A, and 1 μg/mL aprotinin), and kept on ice for 30 min. Cells were lysed by homogenization in a hypotonic buffer, and the cell lysate was sequentially centrifuged at 6000*g* for 10 min. Following the latter centrifugation, the supernatant was removed, and a final NaCl concentration of 0.5 M from a stock solution of 4 M NaCl was reached. The cell lysate was incubated on ice for 30 min. The sample was mixed and brought back to a final NaCl concentration of 0.1 M. The cell lysate was centrifuged for 10 min at 15000*g* and 4 °C. The latter supernatant was removed and recentrifuged at 100000*g* for 60 min in a Beckman ultracentrifuge using SW55 rotor. The amount of protein in the samples described above was determined by the method of Lowry (*25*).

Peptide Synthesis and Binding Assay. Prederivatized plastic rods, active ester, and polypropylene trays were purchased from Cambridge Research Biochemicals (Valley Stream, NY). Peptides were synthesized on solid polypropylene rods as previously described (*26*). For the binding assay, [^{35}S]methionine-labeled proteins from total cell lysate were mixed with an equal volume of 3−6% BSA or 1% gelatin in phosphate-buffered saline and incubated with overlapping hexapeptides covalently linked to polypropylene rods. The peptides and total cell lysate were incubated overnight at 4 °C. The rods were then removed and washed four times in PBS. The bound proteins were eluded by incubating the pins in 1× SDS sample buffer for 60 min at room temperature with shacking.

SDS−PAGE and Western Blotting. Protein fractions (100−150 μL) were resolved via SDS−PAGE using the Laemmli gel system (*27*). Proteins were dissolved in 3× solubilization sample buffer I [62.5 mM Tris-HCl (pH 6.8) containing 2% (w/v) SDS, 10% (w/v) glycerol, and 5% β-mercaptoethanol], and samples were electrophoresed at a constant current. Gel slabs containing the resolved proteins were fixed in 50% methanol and 10% acetic acid. Polyacrylamide gels containing [^{35}S]methionine labeled proteins were exposed to Kodak X-ray film following a 30 min incubation in an Amplify solution (Amersham Inc.). Alternatively, proteins were transferred to a nitrocellulose membrane in Tris-glycine buffer in the presence of 20% methanol for Western blot analysis according to the procedure of Towbin (*28*). The nitrocellulose membrane was incubated in a 5% skim milk/PBS mixture prior to the addition of anti-tubulin monoclonal antibody (0.5 μg/mL in 3% BSA; Amersham Inc.). Following several washing steps with PBS, the nitrocellulose was incubated with peroxidase-conjugated antibody, and immunoreactive proteins were visualized by chemiluminescence using the ECL method (Amersham Inc.).

Protein Purification and N-Terminal Sequencing. The 57 kDa protein was purified using a block of polypropylene pins with one of the affinity binding peptide sequences covalently attached. Briefly, the latter pins were incubated with total cell lysate as indicated above; however, in this case, the carrier substance was gelatin (1%). The bound proteins were eluded in 100 mM phosphate buffer (pH 7.4) containing 2% SDS and 0.1% β-mercaptoethanol. The eluted proteins were precipitated by mixing them with 9 volumes of ice-cold ethanol and incubated at −20 °C. Following a high-speed centrifugation of the latter sample (centrifugation for 15 min at 15000*g* and 4 °C), the precipitated proteins were resuspended in 1% SDS in PBS and mixed with an equal volume of 2× SDS Laemmli sample buffer (*27*). Protein samples were resolved via 10% SDS−PAGE and transferred to PVDF membrane. The migration of the 57 kDa band was visualized by staining the PVDF membrane with Ponceau S. The PVDF membrane containing the 57 kDa band was excised from and submitted to the protein sequencing facility at the Biotechnology Service Centre in Toronto, ON. Amino acid sequencing of peptides was performed according to the method of Edman and Begg (*29*) using an Applied Biosystems gas-phase model 470A sequenator according to the procedure described by Flynn (*30*).

RESULTS AND DISCUSSION

The physiologic function(s) of the ABCB1 linker domain remains a matter of speculation. In this study, it was of

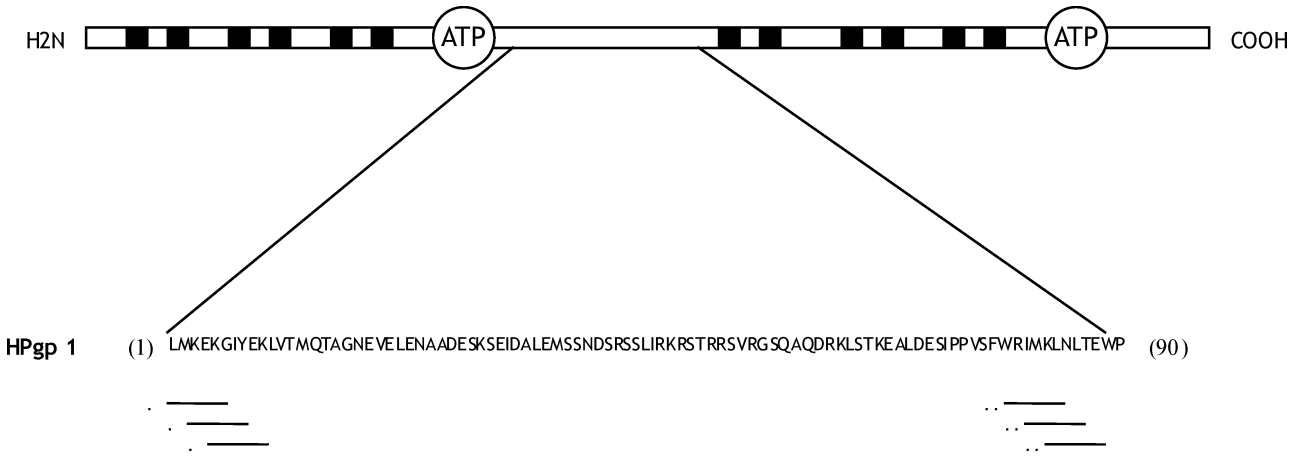

FIGURE 1: P-Glycoprotein-predicted secondary structure and amino acids of the linker domain. A schematic representation of P-gp-predicted secondary structure. The 12 black squares represent the 12 putative transmembrane domains. The two ATP binding domains are represented by two circles in the N- and C-terminal halves of P-gp. The inset represents the linker domain. The amino acid sequence of the linker domains of ABCB1 is indicated as a single-letter amino acid code. The numbers in brackets at the beginning and end of each amino acid sequence of ABCB1 show the length of the linker domains (1−90). The numbered lines underneath the amino acid sequence show the sequence of the overlapping hexapeptides, which differ by one amino acid.

interest to identify cytosolic proteins that interact with the linker domain ABCB1 transporter. Using a novel approach to identifying high-affinity binding sequences (or HABS; E. Georges, manuscript in preparation), a total of 92 overlapping hexapeptides were synthesized to cover the entire linker sequence of ABCB1 (see Figure 1). This approach allows the simultaneous identification of the "high-affinity binding sequences" within the linear sequence of a given protein ("the bait") and interacting proteins ("the capture") from a large mix of proteins. This is achieved experimentally by synthesizing short overlapping peptides on a solid support and allowing such short flexible peptides to interact with a metabolically labeled pool of cellular proteins. Briefly, peptides encoding the entire linker sequence of ABCB1 were synthesized onto plastic rods and then incubated with total cell lysate from CEM/VLB$^{1.0}$ cells metabolically labeled with [^{35}S]-methionine. The bound proteins were then eluded with SDS-containing buffers and resolved via SDS−PAGE (see Experimental Procedures). Figure 2 shows proteins bound to the 92 overlapping hexapeptides from the ABCB1 linker sequence. Three regions in the ABCB1 linker domain [^{617}EKGIYFKLVTM^{627} (LDS$^{617-627}$), ^{657}SRSSLIRKRST-RRSVRGSQA^{676} (LDS$^{657-676}$), and ^{693}PVSFWRIMKLN-LT^{705} (LDS$^{693-705}$)] bound a 57 kDa protein (or P57). Hexapeptides 46−60, 81−89, and 5−9 (Figure 1) bound with decreasing affinities to P57 protein (Figure 2). A peak increase in the intensity of peptide−protein interaction is seen clearly within LDS$^{657-676}$ sequences. Such increase in the level of peptide−protein interaction at a resolution of a single amino acid points to the specificity of this interaction and the applicability of this approach to mapping high affinity binding domains between any two interacting proteins. In addition to P57, peptides 46−60 bound two other proteins with apparent molecular masses of 80 and 30 kDa, but to a lesser extent. It is likely that the latter proteins (80 and 30 kDa) are associated with P57, since these proteins are detected when the intensity of the P57 protein signal is high (Figure 2, peptides 50−56). Comparison of the amino acid sequences of the three polypeptides that bound P57 did not reveal significant sequence homology among the three polypeptides to account for their binding to the same protein. Interestingly, however, the amino acid sequence of the second region (peptides 46−60) encodes protein kinase C consensus sequences (*31*). The third region (peptides 81−89) was shown to encode a protein kinase A site (*32*). Work is ongoing to determine if modifications of such sequences by phosphorylation affect their ability to interact with P57 protein. Such short polypeptides encoding high-affinity signature sequences have been previously described, for example, the Src homology-2 or SH2 domain of Src-family kinases shown to bind tightly to a phosphorylated tyrosine (Y*-EEI) sequence found in the epidermal growth factor receptor and the focal adhesion kinase (*33*).

To determine the identity of P57 protein, sufficient amount of this protein was harvested through the use of several copies of two hexapeptides (^{658}RSSLIR^{663} and ^{669}SVRGSQ^{674}) from the second region of the ABCB1 linker domain, which exhibited the strongest capacity to bind to P57. As expected, the results in Figure 3 show that both hexapeptides bound specifically to the 57 kDa protein. However, both hexapeptides bound another protein with an apparent molecular mass of ∼41 kDa. Interestingly, longer incubation times of the total cell lysate led to an increase in the levels of the 41 kDa protein (Figure 3). We speculate that the 41 kDa band is likely a degradation product of P57 protein due to the addition of 1% gelatin, instead of 3% BSA as a carrier molecule that would allow the isolation of albumin-free P57.

Using multiple copies of the two high-affinity hexapeptides (^{658}RSSLIR^{663} and ^{669}SVRGSQ^{674}) in pulldown assays, considerable amounts of pure P57 protein were easily obtained. Figure 4 shows a Coomassie blue-stained SDS−PAGE gel containing the peptide-captured P57 protein. The purified protein was transferred to a PVDF membrane and stained with Ponceau S to localize the protein. The Ponceau S stained band migrating with the expected molecular mass was cut out and used for direct N-terminal sequencing (*30*). The first seven rounds of Edman degradation showed two sequences, MREVISI and MREIVHI. The two polypeptide sequences differed by only three amino acids (VIS instead of IVH). Comparison of the two sequences with known protein sequences using the FastA protein search engine

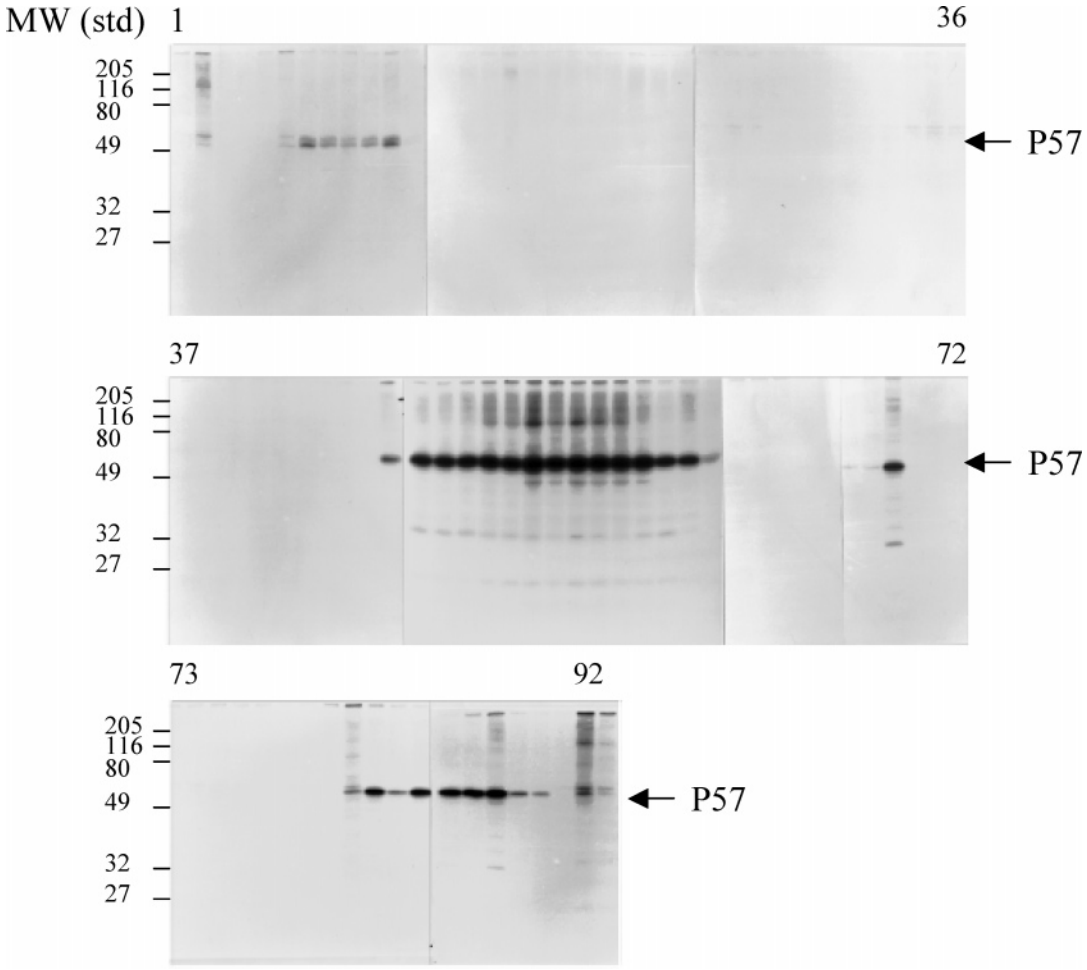

FIGURE 2: Binding of cytosolic proteins to overlapping hexapeptides encoding the ABCB1 linker domain. Overlapping hexapeptides that encode the linker domain of ABCB1 were synthesized on polypropylene rods and used to identify proteins that bind to these peptides. A total of 90 plus two control hexapeptides for ABCB1 were incubated with total cell lysate from CEM/VLB$^{1.0}$ cells metabolically labeled with [^{35}S]methionine (see Experimental Procedures). All bound proteins were eluted from the peptide-coupled rods and resolved via 10% SDS−PAGE. Lanes 1−92 show the [^{35}S]methionine-bound proteins from ABCB1. The migration of the molecular weight markers is shown to the left of the figure.

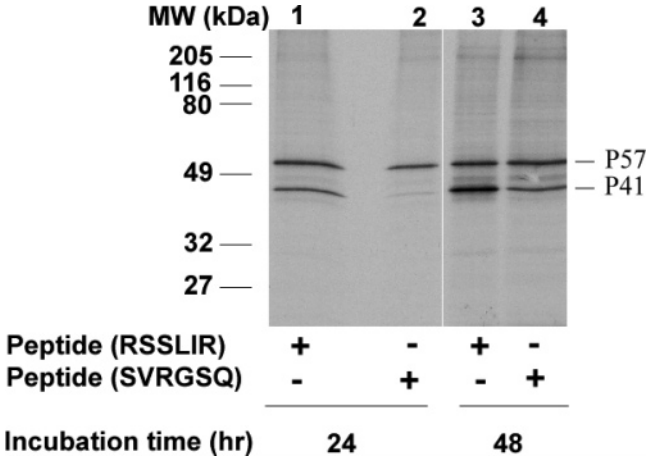

FIGURE 3: Binding of cytosolic proteins to two high-affinity hexapeptides. Two high-affinity binding sequences, ^{658}RSSLIR^{663} and ^{669}SVRGSQ^{674}, from the ABCB1 linker domain were resynthesized and incubated with total cell lysate from CEM/VLB$^{1.0}$ cells metabolically labeled with [^{35}S]methionine following a 24 or 48 h incubation. Bound proteins were eluded from peptide-coupled rods and resolved via 10% SDS−PAGE. The migration of the molecular weight markers is shown at the left.

revealed the first seven N-terminal amino acids of α- and *β*-tubulins. The identification of tubulins, as the 57 kDa protein, was consistent with the apparent molecular mass and the potential degradation products that were observed following long incubation periods (e.g., the 41 kDa polypeptide). The identity of P57 protein as tubulin was confirmed by Western blotting on eluted material probed with anti-α-tubulin and anti-*β*-tubulin monoclonal antibodies. Figure 5 shows the results of the Western blot analysis. Consistent with the sequencing results, both tubulin subunits (α and *β*) were recognized in the lanes containing the hexapeptide-bound proteins, thus establishing the identity of the 57 kDa protein as tubulin.

The physiological significance of the binding of ABCB1 to tubulin is not clear. However, tubulin has been shown to interact with several membrane proteins (*34*−*37*). Therefore, the interaction of ABCB1 with tubulin, and possibly microtubules, is consistent with the membrane−skeleton fence model (*38*). In that model, a small fraction of membrane receptors seem to be fixed to the underlying cytoskeleton (*39*). It is interesting in this respect that the increases in the stability and level of expression of ABCB1 in Rat liver tumors in vivo are associated with similar increases in the stability of several cytoskeleton proteins, including α-tubulin, *β*-actin, and cytokeratins 8/18 (*40*).

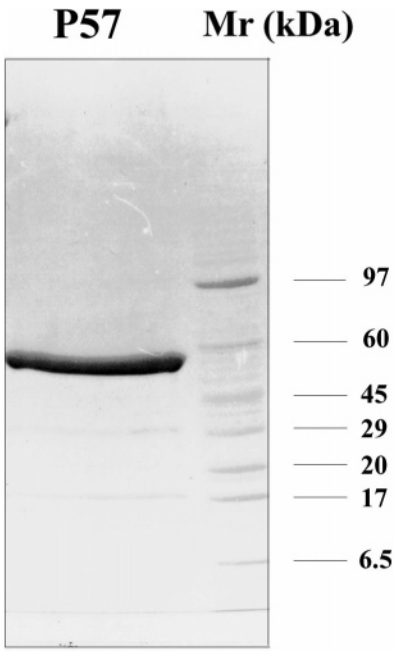

FIGURE 4: Purification of the 57 kDa protein. Total cell lysate was incubated with 50 ABCB1 hexapeptides, ^{658}RSSLIR^{663} and ^{669}SVRGSQ^{674}. Samples containing the 57 kDa protein (P57) from a 100-hexapeptide incubation mix were pooled and resolved via 10% SDS−PAGE. The resolved proteins were transferred to a PVDF membrane and stained with Ponceau S. The migration of the molecular weight markers is shown at the right.

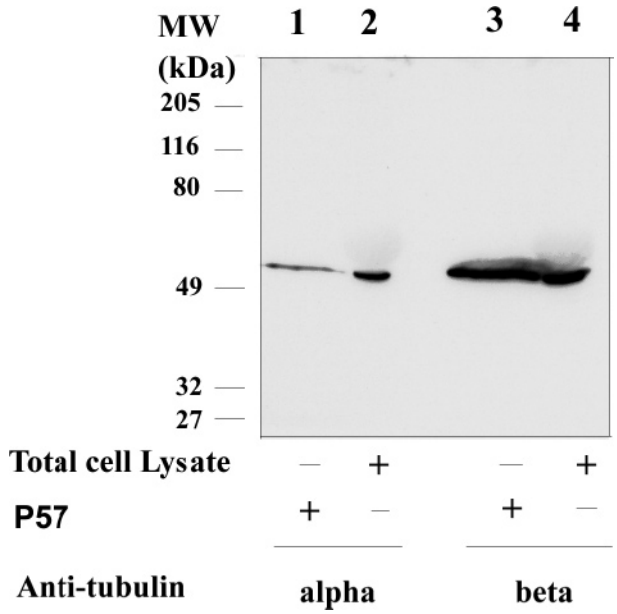

FIGURE 5: Western blot analysis with anti-tubulin monoclonal antibodies. Total cell lysate from CEM/VLB1.0 cells and proteins eluted from the high-affinity binding hexapeptides of the ABCB1 linker domain (P57) were resolved via SDS−PAGE and transferred to a nitrocellulose membrane. One-half of the membrane was probed with anti-α- and anti-*β*-tubulin monoclonal antibodies. The migration of the molecular weight markers is shown at the left.

In addition, the region with the highest affinity for P57 encodes the three putative phosphorylation sites in ABCB1 (*31*). Hence, it is possible that interaction of ABCB1 with tubulin through the linker domain is modulated by phosphorylation of these sites. Mutations of ABCB1 phosphorylation sites within the linker domain were shown not to affect its drug transport function (*22*), while other proposed functions of ABCB1 (e.g., regulator of endogenous chloride channel) were modulated by phosphorylation (*23*). Indeed, a member of the ABC transporter family, CFTR (the cystic fibrous transmembrane regulator), which encodes a similar linker domain was found to colocalize to the microtubule network (*41*). Furthermore, microtubule-dependent acute recruitment of CFTR to the apical plasma membrane of T84 cells was responsive to elevations in intracellular cAMP levels and phosphorylation of the linker domain (*41*). Taken together, the localization of tubulin binding sequences to the linker domain of ABCB1 protein is the first direct evidence for a role of this domain in ABCB1 protein interactions. Moreover, the overlap in ABCB1 phosphorylation with tubulin binding sequences leads one to speculate about the role of phosphorylation as a modulator of binding of ABCB1 to tubulin or microtubule filaments. It is worth noting that selection of tumor cells for resistance to drugs that modulate or affect microtubule integrity often leads to the overexpression of ABCB1. Consequently, it is possible that interaction of ABCB1 with tubulin is part of a signaling mechanism whereby a change to microtubulin integrity leads to enhanced ABCB1 expression as a general defense mechanism. Alternatively, ABCB1−tubulin interaction may be important in determining the protein half-life and turnover. Work is ongoing to examine these possibilities, including the role of ABCB1 phosphorylation in tubulin binding.

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