Vesicular localization of the rat ATP-binding cassette half-transporter rAbcb6

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Abstract

Vesicular localization of the rat ATP-binding cassette half-transporter rAbcb6 is demonstrated by double-labeling with an antibody against the COOH-terminal epitope tag (rAbcb6-V5). Following subcellular fractionation, rAbcb6-V5 was devoid of endogenous human ABCB6 mRNA, was employed for heterologous expression of rAbcb6 bearing a COOH-terminal epitope tag (rAbcb6-V5). Following subcellular fractionation, rAbcb6-V5 was observed as an N-glycosylated protein in fractions enriched with lysosomal/endosomal membrane proteins. Indirect immunofluorescence analyses of rAbcb6-V5 using antibodies against a rAbcb6-specific peptide or against the V5-tag revealed a punctate pattern that was colocalized with lysosome-associated membrane protein 1 (LAMP1), a marker of lysosomes/late endosomes. Substantial colocalization of tagged rAbcb6 with lysosomal/endosomal membrane proteins was confirmed with living, unfixed LoVo cells coexpressing rAbcb6 fused to enhanced green fluorescent protein. Vesicular distribution in LoVo cells was consistent with localization of endogenous rAbcb6 expressed in rat primary hepatocyte cultures or in liver sections, as revealed by overlap of rat LAMP1 with rAbcb6 in double immunofluorescence analyses. Several Abcb6-related half-transporters confer heavy metal tolerance. In LoVo cells might affect sensitivity toward transition metal toxicity. Applying MTT viability assays, we found that expression of either rAbcb6-V5 or untagged rAbcb6 conferred tolerance toward copper, but not to cobalt or zinc. In summary, these results demonstrate that rAbcb6 is a glycosylated protein targeted to intracellular vesicular membranes and suggest involvement of rAbcb6 in transition metal homeostasis.

LoVo; rat hepatocytes; lysosomes; endosomes; glycosylation; copper tolerance

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between individual transporters. Primarily intracellularly, the precise distribution differs between related half-transporters in other species appear to be situated in mitochondria (Atm1p) half-transporter found in Saccharomyces cerevisiae, as well as in animals (4). Finally, rAbcb6 exhibits sequence similarity to the human ABCB7 protein (9), with 41% identity over a 617-amino acid overlap, and to the ABC transporter in mitochondria 1 (Atm1p) half-transporter found in Caenorhabditis elegans, indicating that comparable mechanisms of alleviating heavy metal toxicity exist in fission yeast, as well as in animals (40). Taken together, although rAbcb6-related half-transporters in other species appear to be situated primarily intracellularly, the precise distribution differs between individual transporters.

A major aim of the present study was to clarify the subcellular distribution of rAbcb6. The knowledge of rAbcb6 localization would be expected to provide an important basis for further resolution of its physiological function.

MATERIAL AND METHODS

Culture of human cell lines and primary rat hepatocytes, and preparation of rat tissue sections. The human colon adenocarcinoma line LoVo was purchased from the DSMZ (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany). Human HepG2 cells were kindly provided by T. Gebel (Department of General Hygiene and Environmental Health, University of Göttingen). Both LoVo and HepG2 cells were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum and antibiotics (penicillin, 100 U/ml medium, and streptomycin, 100 μg/ml medium) in an incubator, maintaining 5% CO2 in the atmosphere. Primary rat hepatocytes were isolated from adult male Wistar rats and cultured as described previously (43). Liver tissue was obtained from an adult male Wistar rat and immediately frozen in isopentane. Cryostat sections of 15-μm thickness were prepared on a Leica cryostat microtome. Sections were stored at −80°C until further processing for immunohistochemistry.

Messenger RNA expression analyses. Total RNA was isolated from cultured rat hepatocytes or human cell lines, according to Ref. 8. For Northern blot analyses, 20 μg of total RNA per lane were separated through a 1% formaldehyde/agarose gel. To check for equal loading of lanes, gels were stained with ethidium bromide. RNA was blotted onto Hybond N membranes (GE Healthcare, Freiburg, Germany), and lanes, gels were stained with ethidium bromide. RNA was blotted onto Hybond N membranes (GE Healthcare, Freiburg, Germany), and

For detection of rAbcb6 and human ABCB6 mRNA expression by reverse transcription (RT)-PCR, 1 μg of total cellular LoVo RNA was subjected to RT and subsequent PCR of cDNA using the Titan One Tube RT-PCR Kit (Roche, Mannheim, Germany) and the following primers specific for human ABCB6: 5'-GGATGCCAGCCAGAGCT-3' (hB6rev). RT was performed at 50°C for 30 min and terminated at 94°C for 2 min. Cycling conditions for PCR were as follows: denaturation at 94°C for 15 s, annealing at 60°C for 30 s, and elongation at 68°C for 2.5 min + 5 s/cycle. Final extension was performed at 68°C for 7 min.

rAbcb6- and organellar expression plasmids. A PCR product representing the entire coding sequence of rAbcb6 cDNA, but lacking the stop codon, was cloned into the TOPO TA cloning site of the pcDNA3.1/V5/His vector (Invitrogen, Karlsruhe, Germany), in frame with the COOH-terminal V5 epitope and the polyhistidine tag encoding region, to generate the expression vector pcDNA3.1/rAbcb6/V5/His. rAbcb6 cDNA containing the native stop codon was cloned into the same recipient vector to obtain an expression vector for rAbcb6 without an epitope tag (pcDNA3.1/rAbcb6). Alternatively, rAbcb6 cDNA was amplified with primers introducing restriction enzyme cleavage sites and, following digestion, was ligated into pEGFP-N1 (BD Biosciences, Palo Alto, CA), leading to the expression plasmid pAbcb6-enhanced green fluorescent protein (EGFP). The organellar marker plasmid pDsRed-Mito, yielding a DsRed fusion protein directed to mitochondria via the targeting sequence of cytochrome-c oxidase subunit VIII, was constructed as described previously (28). The plasmid pDsRed-Lyso, which led to expression of DsRed targeted to lysosomes/late endosomes, was constructed accordingly (28) and yielded a protein (Lamp1-DsRed) comprising the rat lysosome-associated membrane protein (Lamp) 1 (including the cytoplasmic tail with the late endosomal/lysosomal targeting sequence [32]) fused to COOH-terminal DsRed.

Transfection of LoVo cells. LoVo cells, ~50% confluent, were transiently transfected with pcDNA3.1/rAbcb6/V5/His or transfected with control vector without rAbcb6 cDNA insert, employing the SuperFect transfection reagent (Qiagen, Hilden, Germany), according to the manufacturer’s protocol. In experiments involving transient LoVo transfection with plasmids yielding fluorescent proteins, the Fugene reagent (Roche) was used, as described previously (36). For stable transfection, LoVo cells were transfected with the pcDNA3.1/rAbcb6/V5/His construct or with the corresponding plasmid yielding rAbcb6 protein without a tag, employing the Effectene transfection reagent (Qiagen). Stably transfected cells were selected and maintained with 300 μg/ml G418 (Calbiochem, Merck Biosciences, Darmstadt, Germany) added to the growth medium. A construct bearing the β-galactosidase gene in-frame with the V5/His epitope coding region (pcDNA3.1/V5/His-Topo/lacZ, Invitrogen) was used to generate stably transfected control LoVo cell lines.

Isolation of subcellular fractions. For isolation of crude membrane fractions from LoVo cells, all steps were performed at 4°C in the presence of 1 mM of the protease inhibitor Pefabloc SC (Roche). Crude membrane fractions were obtained, applying a procedure according to Ref. 39. Cells were homogenized in succrose buffer TES (20 mM Tris, 1 mM EDTA, 254 mM sucrose, pH 7.4; total volume 20 ml) by 10 strokes in a 30-ml Teflon pestle homogenizer, and differential centrifugation was subsequently performed as outlined in Fig. 1. The postnuclear supernatant resulting from centrifugation at 650 g for 10 min (SN1) was subjected to centrifugation at 10,000 g for 10 min, yielding a pellet enriched in mitochondria (see Fig. 3A, fractions 1a and 1b). The resulting supernatant SN2 was further centrifuged at 18,000 g for 30 min. The collected supernatant (SN3) was centrifuged at 48,000 g for 30 min, and the pellet was resuspended in TES (see Fig. 3A, fractions 3a and 3b). The supernatant thereof (SN4) was centrifuged at 205,000 g for 75 min, and the resulting pellet was resuspended in TES (see Fig. 3A, fractions 4a and 4b). The pellet obtained after centrifugation of supernatant SN2 at 18,000 g was resuspended in TES, layered onto a cushion of 20 mM Tris, 1 mM EDTA, 1.12 M sucrose, pH 7.4, and centrifuged at 100,000 g for 1 h. The fraction collected from the interphase was diluted in 10 ml TES, and pelleted at 100,000 g for 1 h. The resulting fraction (see Fig. 3A, fractions 2a and 2b), enriched in lysosomal/late endosomal marker Lamp1, was resuspended in TES. Isolated membrane fractions were stored in liquid nitrogen.

Fractions enriched in lysosomal membranes were further obtained from LoVo cells grown on twenty 58-cm2 culture dishes to 50%
cytchrome-c oxidase subunit IV (20E8-C12, Molecular Probes, Leiden, The Netherlands); monoclonal anti-human LAMPI (BD Biosciences, San Diego, CA); monoclonal anti-EAA1 (early endosome antigen 1); monoclonal anti-BiP/GRP78 (immunoglobulin heavy chain-binding protein/glucose-regulated protein); monoclonal anti-GM130; monoclonal anti-annexin II; monoclonal anti-PECI (peroxisomal monofunctional delta3, delta2-enoyl-CoA isomerase) (all BD Biosciences). Secondary, peroxidase-conjugated antibodies directed against primary polyclonal antibodies (goat anti-rabbit IgG, Sigma no. A0545, diluted 1:10,000) or against monoclonal mouse antibodies (goat anti-mouse IgG, Sigma no. A9309, diluted 1:4,000) were employed for visualization of immunoreactive proteins in conjunction with the enhanced chemiluminescence detection kit (GE Healthcare).

Immunoprecipitation. LoVo cells were harvested 3 days after transient transfection. Following homogenization, samples were cleared of nuclei by centrifugation at 650 g, and the postnuclear fraction was used for immunoprecipitation. Aliquots containing 300 μg protein (in a volume of ~50 μl) were incubated with an equal volume of lysis buffer (300 mM NaCl, 100 mM HEPES, 2% Triton X-100, 4 mM Pefabloc, pH 7.4) for 1 h at 4°C. Subsequent to centrifugation of samples at 14,000 g for 30 min, the resulting supernatant was incubated for 2 h at 4°C under agitation, either with the polyclonal antibody against rAbcb6 (1:100) or with the corresponding preimmune serum obtained from the rabbit before immunization (1:100). A suspension of preswollen protein A sepharose (GE Healthcare), containing 3.75 mg sepharose/50 μl, was added to the samples in an equal volume, and precipitation of immune complexes was performed at 4°C for 90 min under agitation. Subsequently, samples were spun for 1 min at 10,000 g, supernatants were discarded, and the sepharose pellet was washed three times with 10 mM NaCl, 50 mM HEPES, 0.1% Triton X-100, pH 7.4; twice with 150 mM NaCl, 50 mM HEPES, 0.1% Triton X-100, pH 7.4; and once with 150 mM NaCl, 50 mM HEPES, 0.1% Triton X-100, 0.1% SDS, pH 7.4. Finally, protein bound to the sepharose was eluted by incubation with 2% Laemmli sample buffer (25) for 5 min at 95°C. Following centrifugation at 10,000 g for 30 s, 10 μl of each supernatant were resolved by SDS-polyacrylamide gel electrophoresis with gels containing 7.5% acrylamide and blotted onto polyvinylidene difluoride membranes, as described above. Immunoprecipitated protein was detected in Western blots using V5 antibody (1:5,000) as the primary antibody.

Immunofluorescence analyses and fluorescent protein expression analyses. LoVo cells stably transfected with rAbcb6 or rAbcb6-V5 expression vectors were grown on coverslips to ~70% confluence. Primary rat hepatocytes were cultured on coverslips for 2 days. Rat liver tissue sections were prepared as described above. Specimens were washed with PBS and subsequently fixed in prechilled methanol for 10 min at −20°C. All further incubation steps were performed at 20°C. Cells were permeabilized with 0.1% Triton X-100 in PBS for 5 min and blocked with 10% normal goat serum in PBS for 30 min. Specimens were then briefly rinsed in PBS and subsequently incubated for 1 h with the primary antibody, diluted in 0.5% BSA/1.5% normal goat serum in PBS (the dilution being 1:50 to 1:100 for the unpurified peptide-specific antisera against rAbcb6, 1:10 for the purified rAbcb6 antibody, 1:10 for the monoclonal V5-antibody, 1:25 for monoclonal anti-human-LAMPI, and 1:200–1:300 for monoclonal anti-rat-Lamp1; Stressgen, Ann Arbor, MI). After rinsing in PBS, specimens were incubated for 1 h with appropriate secondary anti-
bodies. Fluorescein isothiocyanate-conjugated affinity-purified anti-rabbit IgG from goat (Roche), diluted 1:500, or Alexa Fluor 488-conjugated goat anti-rabbit IgG (Invitrogen), diluted 1:500–1:2,000, was used for the detection of polyclonal primary antibodies. Tetra-ethylrhodamine isothiocyanate-conjugated anti-mouse IgG from goat (Sigma no. T6653), diluted 1:50, or Alexa Fluor 546-conjugated goat anti-mouse IgG1 (Invitrogen), diluted 1:2,000, was employed for the detection of primary monoclonal antibodies. After washing in PBS, the specimens were mounted in glycerol or in mounting medium (Vectorshield, Vector Laboratories, Burlingame, CA) and examined with a Zeiss fluorescence microscope. Fluorescence of rAbcb6-EGFP and of red fluorescent organelle marker proteins (Mito-DSRed and Lamp1-DsRed) was observed 2–3 days after transfection via fluorescence microscopy in unfixed LoVo cells that had been grown on Lab-Tek II chamber slides (Nalgene Nunc, Kamstrup, Denmark) and transfected with red or green fluorescent protein expression vectors, as described previously (36).

**RESULTS**

**Lack of ABCB6 mRNA expression in the human colon adenocarcinoma cell line LoVo.** One of the strategies pursued by us for investigation of the subcellular distribution of the rAbcb6 protein involved the expression of rAbcb6 as an epitope-tagged fusion protein and detection of the fusion protein via a tag-specific antibody. To select a recipient cell line suitable for overexpression of epitope-tagged rAbcb6 and for later functional experiments, transcript expression in different cell lines was examined by RT-PCR and Northern blot analyses. Several human cell lines, e.g., the human hepatoma cell line HepG2 (14) or the human neuroblastoma cell line SKNSH (data not shown) expressed endogenous human ABCB6 mRNA. Northern blot analysis demonstrated that the size of the human transcript was comparable to the size of the transcript found in primary rat hepatocytes (3.4 kb, Ref. 14, Fig. 2). Nevertheless, we found the human colon carcinoma cell line LoVo to be devoid of ABCB6 mRNA in both Northern blot (Fig. 2) and RT-PCR analyses (data not shown).

**Detection of rAbcb6-V5 fusion protein in subcellular fractions of transiently transfected LoVo cells.** LoVo cells were used as recipient cells for transient and stable transfection with a vector expressing a rAbcb6 fusion protein (rAbcb6-V5). This fusion protein comprised rAbcb6 extended by a COOH-terminal peptide containing the viral V5 epitope and terminal histidine residues (His6). Employing a commercially available monoclonal antibody directed against the V5-epitope, we were able to probe for the fusion protein in immunoblots of subcellular fractions obtained by differential centrifugation (as outlined in Fig. 1 and in the MATERIAL and METHODS section). In transiently transfected LoVo cells, substantial rAbcb6-V5 protein expression was observed within 3 days after transfection (Fig. 3A). The rAbcb6-V5 fusion protein exhibited an electrophoretic mobility corresponding to ~80–97 kDa (Fig. 3B). No substantial immunoreactivity was found in the postnuclear pellet sedimented at 10,000 g (Fig. 3A, fraction 1b), which was enriched in mitochondria, as demonstrated by the presence of the cytochrome-c oxidase subunit IV (Fig. 3B). However, major immunoreactivity against V5 was observed in the postmitochondrial pellet purified on a sucrose cushion (Fig. 3, A and B, fraction 2b). This fraction was enriched in human LAMP1, a marker of late endosomes and lysosomes, and in annexin II, a protein recruited to endosomal vesicles and to actin assembly sites at the plasma membrane (38) (Fig. 3B). The fractions 3b and 4b had been sedimented at 48,000 g and 205,000 g, respectively, and thus contained lighter particles than fractions 1b and 2b. Nevertheless, only fraction 3b exhibited slight immunoreactivity against the V5-epitope compared with samples of control-transfected cells (Fig. 3A).

To further define the subcellular localization of the epitope-tagged rAbcb6 protein in transiently transfected LoVo cells, enrichment of lysosomes was performed according to Ref. 33, and rAbcb6-V5 immunoreactivity was compared with organelle marker immunoreactivity, using a panel of antibodies against organelle markers (Fig. 4). The rAbcb6-V5 fusion protein was found to be concentrated in the fractions 1 and 4, representing the postmitochondrial supernatant (fraction 1) and the purified lysosomal membrane fraction (fraction 4). However, the mitochondrial marker cytochrome-c oxidase, the peroxisomal marker PECI, the Golgi marker GM130, or the endoplasmic reticulum marker BiP/GRP78 were not coenriched with rAbcb6-V5. Rather, major rAbcb6-V5 immunoreactivity was found in the fractions enriched in EEA1 (a marker of early endosomes), in LAMP1 (lysosomal/late endosomal marker), or in annexin II (Fig. 4).

**V5-epitope-tagged rAbcb6 represents an N-glycosylated protein.** In immunoblots of cell fractions obtained from rAbcb6-V5-transfected LoVo cells, we found immunoreactive protein (V5-tagged rAbcb6) to exhibit an electrophoretic mobility corresponding to ~80–97 kDa. Immunoreactivity presented either multiple protein bands (Fig. 3A) and/or a smeared broad region (Fig. 4) rather than a single sharply focused band. This is a pattern typically observed with glycosylated proteins (23). We, therefore, examined whether the
rAbcb6-V5-fusion protein concentrated in the fraction enriched with lysosomal membranes (Fig. 4, fraction 4) might be N-glycosylated. Digestion with protein N-glycosidase F led to a shift and focusing in gel mobility of ∼77–80 kDa, and most likely representing the unglycosylated core protein. Taking into account that the V5-epitope tag of the rAbcb6 fusion protein would be expected to add up to ∼5 kDa to the core protein, the electrophoretic mobility of the unglycosylated, untagged rAbcb6 would be estimated to correspond to an even lower molecular mass. This would differ from the molecular mass calculated from the deduced rAbcb6 amino acid sequence, amounting to 93.3 kDa (20). A similar discrepancy has been observed for other ABC half-transporters, e.g., for Abcb9, and may be due to aberrant behavior of partly hydrophobic proteins in an aqueous environment (42). Nevertheless, the deglycosylation experiments support the conclusion that rAbcb6-V5 was indeed expressed in LoVo cells as an N-glycosylated protein.

Distribution of epitope-tagged rAbcb6 (rAbcb6-V5 and rAbcb6-EGFP) in transfected LoVo cells. Due to its V5-tag, the rAbcb6-V5 fusion protein could be detected in transfected LoVo cells with the monoclonal antibody against the V5-epitope. To provide an additional tool for detection of native rAbcb6, as well as epitope-tagged half-transporter, an rAbcb6-peptide-specific polyclonal antiserum was raised in rabbits. The rAbcb6 region corresponding to the peptide designed for immunization was expected to reside outside one of the hy-
drophobic putative transmembrane segments of the protein. Immunoprecipitation experiments performed with lysates of transfected LoVo cells demonstrated that both the rAbcb6-peptide-specific antibody and the anti-V5 antibody bound to the same protein. Following immunoprecipitation by the peptide-specific rAbcb6 antibody, but not by corresponding preimmune serum, the expected rAbcb6-V5 protein band was detected by immunoblot analysis, as outlined in Figs. 3 and 4. The arrow denotes the focusing in electrophoretic mobility following deglycosylation.

To further investigate subcellular distribution of rAbcb6, immunofluorescence analyses were performed on stably transfected LoVo cells expressing rAbcb6-V5 (Fig. 7). Indeed, methanol-fixed, rAbcb6-V5-expressing cells exhibited a distinct, punctate pattern of immunoreactivity, often in the vicinity of the nucleus, which was reminiscent of vesicular structures (Fig. 7, C, D, E, F, I). This distribution differed from fluorescence pattern observed in LoVo cells transfected with the control vector pcDNA3.1/V5/His-TOPO/lacZ and thus expressing V5-epitope-tagged β-galactosidase (Fig. 7H). Marked rAbcb6-V5-dependent immunofluorescence was not observed at the cell margins or plasma membrane, suggesting that the major portion of rAbcb6-V5 was associated with intracellular structures. Comparable punctate patterns of immunofluorescence for the rAbcb6-V5 fusion protein were demonstrated, both with the rAbcb6-peptide-specific antibody (Fig. 7, C, E, F, I) and with the monoclonal V5-antibody (Fig. 7, D and F). Double immunofluorescence experiments were performed in which the fixed LoVo cells were incubated with the polyclonal peptide-specific antibody against rAbcb6, together with the monoclonal antibody against the V5-epitope, and subsequently developed with appropriate, differently labeled secondary antibodies. In these experiments, both primary antibodies (anti-V5 and anti-rAbcb6) reacted with the same structures (Fig. 7, E and F), supporting the usefulness of the peptide-specific antibody for detection of rAbcb6 in immunofluorescence procedures. In LoVo cells stably transfected with an expression plasmid yielding untagged rAbcb6, immunofluorescence experiments with the antibody against rAbcb6 revealed a distribution pattern that was in accordance with the distribution of V5-tagged rAbcb6 (data not shown). Double immunofluorescence analyses of rAbcb6-V5 expressing cells involving both the polyclonal antibody against rAbcb6 and a monoclonal antibody against human LAMP1 showed punctate immunoreactivity that indicated rAbcb6-V5 protein colocalization with the lysosomal/late endosomal marker protein LAMP1 (Fig. 7, I and J).

To further specify rAbcb6 fusion protein localization in living cells and to avoid cell fixation/permeabilization during specimen preparation as a possible perturbing factor in localization studies, we coexpressed rAbcb6 fused to EGFP (rAbcb6-EGFP) with red fluorescent organelle marker protein in transiently transfected LoVo cells. As observed in the unfixed cells, the distribution of green fluorescent rAbcb6-EGFP showed a marked overlap with the localization of the red fluorescent lysosomal/late endosomal marker (rat Lamp1-DsRed fusion protein), confirming colocalization between tagged rAbcb6 and lysosomal/endoosomal marker (Fig. 8). However, the red fluorescent mitochondrial marker (DsRed targeted to mitochondria, Mito-DsRed) exhibited a pattern of distribution distinct from that of rAbcb6-EGFP (Fig. 8). Additionally, consistent patterns of intracellular localization of green fluorescent rAbcb6-EGFP were observed in other transfected cell lines, e.g., in TM3, a murine cell line that displays characteristics of Leydig cells and also expresses endogenous murine Abcb6 (data not shown). Thus these results support the conclusion that two rAbcb6 fusion proteins containing different COOH-terminal tags (rAbcb6-V5 and rAbcb6-EGFP) and showing colocalization with the late endosomal/lysosomal marker proteins LAMP1/rat Lamp1-DsRed were associated with vesicular structures.

Subcellular distribution of endogenous rAbcb6 in primary cultures of rat hepatocytes and in rat tissue sections. We previously demonstrated that primary rat hepatocytes, cultured under serum-free conditions for up to 4 days, displayed stable rAbcb6 mRNA expression as detected by Northern blot analyses (20), which is consistent with the rAbcb6 mRNA expres-

![Fig. 5. Deglycosylation of the rAbcb6-V5-fusion protein expressed in LoVo cells by digestion with N-glycosidase F (PNGase F). Twenty-microgram samples of a fraction enriched in lysosomal membranes (corresponding to fraction 4 in Fig. 4) and obtained from LoVo cells transiently transfected with the pcDNA3.1/rAbcb6/V5/His construct) were digested with N-glycosidase F, as described in MATERIAL AND METHODS.](http://ajpcell.physiology.org)
sion demonstrated for primary rat hepatocytes in Fig. 2. To examine the distribution of endogenously expressed rAbcb6 protein in rat hepatocytes, the peptide-specific antibody against rAbcb6 and an antibody against rat Lamp1 were used in double immunofluorescence analyses. Immunofluorescence for endogenous rAbcb6 in cultured rat hepatocytes as well as in rat liver tissue sections revealed a punctate pattern that overlapped with distribution of rat Lamp1 (Fig. 9). Thus the localization of endogenous rAbcb6 in primary rat hepatocytes and in the liver tissue context was in accordance with the distribution of the tagged rAbcb6 that was heterologously expressed in cell lines (e.g., in LoVo cells).

Expression of rAbcb6 in LoVo cells confers tolerance to copper ions. Since the rAbcb6-related half-transporters HMT1 and CeHMT-1 have been demonstrated to confer heavy-metal tolerance in the fission yeast (34) and in Caenorhabditis elegans (40), respectively, we investigated whether rAbcb6 might alter susceptibility toward transition metal toxicity. We,
therefore, compared LoVo cells stably transfected with a plasmid expressing the rAbcb6-V5 protein or the untagged rAbcb6, to parental LoVo cells and LoVo cells transfected with a plasmid expressing a fusion protein (β-galactosidase-V5-His) that contained the same COOH-terminal tag as rAbcb6-V5. Toxicity of transition metal salts (CuSO₄, CoCl₂, or ZnSO₄) was assessed by the MTT test, in which the reduction of a tetrazolium salt to a blue formazan is determined as a measure of cell viability. Cells expressing either V5-epitope-tagged or native rAbcb6 were more tolerant toward CuSO₄ toxicity than control-transfected or parental LoVo cells (Fig. 10, A and B). Fifty percent of maximal MTT conversion were obtained at 160 μM CuSO₄ for control LoVo cells and at 210–220 μM CuSO₄ for rAbcb6- or rAbcb6-V5-expressing cells (Fig. 10A), resulting in ~1.4-fold tolerance toward CuSO₄ in the transporter-transfected cells. On the other hand, transfection of rAbcb6/rAbcb6-V5 did not lead to tolerance toward the other transition metal salts CoCl₂ (Fig. 10C) or ZnSO₄ (data not shown). Thus expression of rAbcb6 or rAbcb6-V5 in the LoVo cell line, which we had found to be devoid of endogenous human ABCB6 mRNA, specifically conferred tolerance toward copper-dependent toxicity.

**DISCUSSION**

In the present study, the human adenocarcinoma cell line LoVo, which lacked expression of endogenous human ABCB6 mRNA, was employed as a recipient line for transfection of rAbcb6 expression plasmids, yielding rAbcb6 or rAbcb6-epitope-tagged fusion proteins (rAbcb6-V5 or rAbcb6-EGFP), respectively. Subcellular fractionation, indirect immunofluorescence analyses, and determination of fluorescent rAbcb6-EGFP distribution support the conclusion that rAbcb6 is localized to vesicular (lysosomal/endosomal) structures. The relevance of this distribution for endogenously expressed rAbcb6 was demonstrated by immunofluorescence analyses of cultured primary rat hepatocytes and of rat liver tissue sections. In addition, expression of rAbcb6 in LoVo cells led to tolerance toward copper-dependent toxicity compared with wild-type or control-transfected counterparts, thus supporting the notion that rAbcb6 may play a role in transition metal homeostasis.

**Intracellular localization of rAbcb6.** The rAbcb6 protein belongs to a subgroup of related ABC half-transporters within the [MDR/TAP (transporter associated with antigen processing)] cluster of ABC proteins, which includes the *Saccharomyces cerevisiae* Atl1p, human ABCB7, the fission yeast HMT1 protein, and CeHMT-1. Although the members of this group reside in intracellular membranes, the subcellular localization is heterogenous: human ABCB7 and Atl1p are associated with the mitochondrial inner membrane (9, 27), whereas HMT1 is targeted to the vacuolar membrane (34, 35). Heterologous expression of CeHMT-1 in *S. pombe* revealed a vacuolar membrane localization also for CeHMT-1 (40). Our findings indicating the lysosomal/late endosomal distribution of rAbcb6 are in line with the vacuolar membrane localization.
of HMT1, since the yeast vacuole is regarded as being analogous to lysosomes in higher eukaryotic cells (41).

Interestingly, previous studies concerning human ABCB6 (also termed MTABC3) have suggested mitochondrial localization (31) or a mitochondrial outer membrane localization (24). However, the results presented for rAbcb6 in the present study challenge the notion that mitochondria represent the general target organelle for mammalian Abcb6. Subcellular fractionation, as well as fluorescence microscopy of transfected LoVo cells expressing either rAbcb6-V5 or rAbcb6-EGFP fusion proteins, pointed to a vesicular (lysosomal/endosomal) distribution. This was not due to the choice of the epitope tag, since differently tagged rAbcb6 proteins were colocalized with lysosomal/late endosomal markers (human LAMP1/rat Lamp1-DsRed). Moreover, this localization was not confined only to the LoVo cell system (that was devoid of endogenous human ABCB6 expression). Transfected TM3 cells that express endogenous murine Abcb6 displayed a comparable pattern of rAbcb6-EGFP distribution. Finally, a consistent pattern of endogenous rAbcb6 localization (with rat Lamp1 colocalization) was observed in primary rat hepatocyte cultures and in rat liver tissue sections. The presence of putative lysosomal/endosomal sorting sequences (as reviewed by Ref. 6) within the rAbcb6 primary amino acid sequence is in accordance with lysosomal/endosomal distribution. In particular, a tyrosine-based putative sorting signal of the YXXØ type (in which Ø represents an amino acid with a bulky/hydrophobic side chain) resides within the COOH-terminal region of rAbcb6 (YAEM). Its functional relevance, however, remains to be investigated in further studies.

Deglycosylation experiments performed with V5-tagged rAbcb6 support the conclusion that rAbcb6 is an N-glycosylated integral membrane protein. Extensive glycosylation is typical of lysosomal/late endosomal membrane proteins such as the Lamp proteins, and a lysosomal luminal coat of carbohydrates has been proposed to protect lysosomal membrane proteins against degradation by proteases (18). As lysosomes may fuse with different cellular membranes [endosomes, autophagosomes, plasma membrane (30)], it is conceivable that, in the course of membrane trafficking, a fraction of rAbcb6 might appear in the plasma membrane. However, the fluorescence microscopy procedures performed in the present study with fixed and living transfected LoVo cells or with fixed rat hepatocyte/rat liver specimens are in line with a primarily intracellular (vesicular) localization.
Implications for rAbcb6 function. Several rAbcb6-related half-transporters are involved in metal homeostasis or metal tolerance (exemplified by the participation of Atm1p and ABCB7 in maturation of cytosolic Fe/S cluster proteins, or by the contribution of HMT1 or CeHMT-1 to heavy metal tolerance). Considering that HMT1 appears to confer tolerance toward heavy metal toxicity by transporting phytochelatin-metal complexes into the yeast vacuole (34, 35), it is tempting to speculate that rAbcb6, associated with lysosomal/late endosomal membranes, may have a similar function in translocation of transition metal complexes into the interior of acidic vesicles.

Several transition metals (e.g., iron, copper, zinc, cobalt) are required to fulfill essential cellular functions as trace elements. However, in amounts exceeding those to maintain cellular functions, metal-dependent toxicity may occur. The tendency of iron or copper to participate in redox reactions is the basis for their presence in enzymes catalyzing single-electron transfer reactions. However, Fe^{2+}/Cu^{2+}-dependent redox-reactivity may also contribute to the generation of reactive oxygen species. Therefore, homeostasis of these transition metals is connected to redox homeostasis and must be tightly controlled, e.g., by the presence of iron- or copper-binding molecules and by the activity of membrane-situated transporters.

In the present study, we investigated whether expression of rAbcb6 following transfection of LoVo cells, a cell line we found to be devoid of endogenous human ABCB6 mRNA expression, might confer tolerance to particular transition metals. Interestingly, LoVo clones expressing rAbcb6 exhibited tolerance toward copper (applied as CuSO₄). By contrast, we were not able to observe a comparable tolerance toward salts of other biologically important transition metals such as zinc (ZnSO₄) or cobalt (CoCl₂), indicating a rAbcb6-dependent mechanism of specific cellular protection against toxic effects of copper.

On the one hand, tolerance of LoVo cells toward copper might be explained by sequestration of a copper-binding complex, e.g., involving amino acids or peptides, into endosomes/lysosomes. Thus rAbcb6 may participate in intracellular copper trafficking. Numerous reports exist that establish a link between lysosomal function and cellular trafficking/detoxification of copper. Several transition metals, including nickel, palladium, platinum, copper, silver, and gold, are accumulated in lysosomes (5) and may, in part, be detoxified via lysosomes. During copper overload, hepatocellular excretion into the bile via secretory lysosomes appears to represent a major pathway of detoxification (15). Noteworthy, an ATP-dependent trans-

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Fig. 10. Alleviation by rAbcb6 of Cu²⁺-dependent toxicity. The sensitivity of stably transfected LoVo cells toward CuSO₄- and CoCl₂-dependent toxicity was determined via the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) conversion assay. LoVo cells were seeded at the same density, and the MTT test performed as described in MATERIAL AND METHODS. Data represent mean values ± SD of n independent experiments conducted in multiwell plates, using six parallel wells per salt concentration in each experiment. A: C-V5 denote control cells transfected with the control pcDNA3.1/V5/His-TOPO/lacZ expression vector; ♦ represent cells transfected with the vector expressing native rAbcb6; ● represent cells transfected with the vector expressing the epitope-tagged rAbcb6 (rAbcb6-V5). A: n = 11 (C-V5, rAbcb6), n = 7 (rAbcb6-V5). Significantly different from C-V5 values: *P < 0.05; **P < 0.01; ***P < 0.001; Student’s t-test for unpaired values. B: comparison of MTT conversion at 200 μM CuSO₄ between nontransfected parental cells (LoVo-P), control-transfected cells (C-V5), and cells transfected with a rAbcb6 expression plasmid (rAbcb6/rAbcb6-V5). n = 3 (LoVo-P); n = 11 (C-V5, rAbcb6); n = 7 (rAbcb6-V5). **Significantly different from LoVo-P and C-V5, Student’s t-test for unpaired values, P < 0.001. C: comparison of sensitivity toward CoCl₂. n = 5 (Co-V5); n = 4 (rAbcb6); n = 3 (rAbcb6-V5).
port mechanism specific for copper in the presence of glutathione has been characterized previously on isolated hepatocyte lysosomes (17). However, to our knowledge, the molecular identity of this transporter has not yet been resolved. Known proteins contributing to copper trafficking include Ctr1, which is involved in copper uptake at the plasma membrane (26), and the Wilson disease protein ATP7B, a P-type ATPase, that seems to play a role in biliary copper excretion, possibly by mediating copper transport into the lumen of late endosomes (16). Finally, a novel transporter has been described (Crt2) that appears to enable transport of copper out of lysosomes (37). Mediation of the translocation of copper or a copper complex into the lysosomal interior (as hypothesized for rAbcb6) would be expected to contribute not only to copper sequestration, but also to the establishment of copper stores, which might be used for mobilization of copper required for the assembly of copper-containing enzymes.

Alternatively, tolerance of LoVo cells toward copper toxicity might reflect a mechanism other than direct transport of a copper complex by rAbcb6. Heterologous expression of human ABCB6 protein in mutant Saccharomyces cerevisiae cells that were partially defective in Atm1p function resulted in reversal of the mutant phenotype (31), leading particularly to an alleviation of mitochondrial iron overload and of mitochondrial DNA damage, implying a role of ABCB6 in iron homeostasis (31). Intriguingly, yeast cells exhibiting disruption of the Atm1 gene have been reported to be more sensitive to copper toxicity (2). Therefore, mitochondrial Atm1p protein and vesicular rAbcb6, although situated in different intracellular compartments, may fulfill related functions. Notably, multiple connections exist between iron and copper metabolism (reviewed by Refs. 3, 13). For example, the plasma cupro-enzyme ceruloplasmin catalyzes the oxidation of ferrous iron to Fe3+ (a prerequisite for iron binding to transferrin), and is thus involved in iron mobilization out of storage compartments, e.g., from the liver. Both iron and copper ions have the ability to participate in one-electron redox reactions. In particular, both Fe2+ and Cu+ may lead to the generation of highly reactive hydroxyl radicals by transformation of hydrogen peroxide via the Fenton reaction and may, therefore, contribute to oxidative stress. Thus rAbcb6 might prevent copper-dependent toxicity indirectly by alteration of iron homeostasis and/or by preventing damage that would result from the iron- or copper-dependent generation of reactive oxygen species.

Further studies are required to clarify the exact nature of rAbcb6 substrates. However, a function in transition metal homeostasis would also imply a pivotal role in the regulation of the cellular redox status. Taken together, results presented in our study demonstrate that rAbcb6 is a glycosylated protein targeted to vesicular membranes belonging to lysosomal or endosomal compartments and are in line with the involvement of rAbcb6 in cellular transition metal homeostasis.

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