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Multidrug resistance protein 1 is not associated to detergent-resistant membranes

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Abstract

Multidrug resistance protein 1 (MRP1) is a member of the ATP-binding cassette superfamily. Using the energy provided by ATP hydrolysis, it transports a broad spectrum of substrates across the plasma membrane, including hormones, leukotriene C_4, bile salts, and anti-cancer drugs.

Recent works have suggested that P-glycoprotein is associated to cholesterol and sphingolipid-rich membrane microdomains and that cholesterol upregulates its ATPase and drug transport activities. Confocal microscopy experiments and Triton X-100 extraction of detergent-resistant membranes provide evidence that MRP1 is not located in raft-like structures and that its activity is downregulated by cholesterol. The data are discussed in terms of cholesterol–protein interaction and topology.

Keywords: MRP1; Cholesterol; Detergent-resistant membrane; Transport activity; ATPase activity; ATP-binding cassette (ABC)

A major obstacle to chemotherapy is the emergence of a resistance to anti-cancer drugs associated to the overexpression in the plasma membrane of drug transporters. These proteins reject a broad spectrum of chemotherapeutic agents out of the cells and are responsible for the multidrug-resistant (MDR) phenotype in human cells. Most of these proteins belong to the ATP-binding cassette (ABC) superfamily and use the energy provided by ATP hydrolysis to export cytotoxic drugs outside the cells [1].

P-glycoprotein (Pgp), the first discovered member of the ABC superfamily [2], extrudes from the cell a wide variety of cytotoxic drugs. Recent works have suggested the association of Pgp to cholesterol and sphingolipid-rich membrane microdomains called detergent-resistant membranes (DRMs) [3,4]. Inhibition of Pgp transport and ATPase activities by cholesterol-depleting agents like methyl-β-cyclodextrin (MβCD) [5,6] supports this view. Moreover, cholesterol upregulates Pgp ATPase activity and drug transport in proteoliposomes and cell systems [5,7,8].

The main objective of the present work is to determine whether other ABC transporters are associated to cholesterol and sphingolipid-rich structures and whether cholesterol modulates their ATPase and transport activities. Multidrug resistance protein 1 (MRP1) is another protein

Abbreviations: ABC, ATP-binding cassette; AMP, adenosine monophosphate; ATP, adenosine triphosphate; CFTR, cystic fibrosis transmembrane conductance regulator; CRAC, cholesterol recognition/interaction amino acid consensus; DRM, detergent-resistant membrane; HEK, human embryonic kidney; LTC_4, leukotriene C_4; MβCD, methyl-β-cyclodextrin; MDR, multidrug resistance; MRP, Multidrug resistance protein; NBD, nucleotide-binding domain; Pgp, P-glycoprotein; SSD, sterol sensing domain; TMD, transmembrane domain.

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belonging to this large superfamily of ABC proteins [1]. It transports a broad range of substrates across the plasma membrane [9]. It is an integral membrane protein made of three transmembrane domains (TMDs) and two cytosolic nucleotide-binding domains (NBDs) [1]. Drug binding sites are located into the transmembrane domains [10].

Our experiments carried out on human embryonic kidney cells (HEK-293) overexpressing MRP1 revealed that MRP1 is not located in DRMs. After Triton X-100 extraction of DRMs, MRP1 did not co-localize with Flotillin-1, a known DRM marker protein [11]. Confocal microscopy experiments confirmed distinct localizations of MRP1 and GM1, a typical DRM ganglioside [12]. Experiments carried out on MRP1 proteoliposomes containing increasing amounts of cholesterol revealed that MRP1 drug transport and ATPase activity are downregulated by cholesterol.

Materials and methods

Materials. [^{3}H]LTC$_4$ was purchased from Perkin-Elmer. Asolectin was from Avanti. LTC$_4$, methyl-β-cyclodextrin and cholesterol were from Sigma–Aldrich. Antibodies: MRPm6 (Chemicon International), anti-Flotillin-1 (BD Biosciences), Alexa Fluor 488 and cholera toxin Alexa Fluor 594 conjugate (Molecular Probes), anti-cholera toxin (Calbiochem).

Cell culture. Human embryonic kidney cell line (HEK-293), stably transfected to overexpress MRP1 (HEK-293-MRP1), were grown as adherent monolayer cultures in Dulbecco's modified Eagle's medium (DMEM, Gibco-BRL) supplemented with 10% foetal bovine serum (Gibco-BRL), 2% L-glutamine (Gibco-BRL), and 1% penicillin/streptomycin/fungizone (Gibco-BRL) at 37 °C in a humidified atmosphere (99%) of 5% (v/v) CO_2.

Triton X-100 extraction of DRMs. Extraction of DRMs was performed on HEK-293-MRP1 cells. Cells were washed three times in PBS buffer and resuspended in 1 ml ice-cold MNE buffer (MES 50 mM, pH 6.5; NaCl 150 mM; EDTA 5 mM; Triton X-100 1% (Sigma–Aldrich); anti-proteases (Complete, Roche): one tablet in 50 ml). After incubation (15 min at 4 °C), homogenization with a potter (five times), and centrifugation (2 × 10 min, 5000 rpm), DRMs were mixed with an equal volume sucrose 80%, overlaid by a discontinuous sucrose gradient 30–5% and ultracentrifuged overnight. The gradient was fractionated and these fractions were run on a polyacrylamide SDS–PAGE gel 7.5% analysed by Western blot (antibodies: MRPm6 for MRP1 and anti-Flotillin-1 for DRMs).

Confocal microscopy. Confocal microscopy was performed on 5×10^5 HEK-293-MRP1 cells seeded in a 24-well plate on coverslips coated with polylysine. When the cells had grown to confluence, they were washed once in PBS containing 1% BSA for 10 min. Cells were incubated 30 min with the anti-GM1 cholera toxin (subunit B) Alexa Fluor 594 conjugate, washed 10 min and then incubated 30 min with anti-cholera toxin antibody. After 10 min washing, cells were fixed with 2% paraformaldehyde in PBS for 10 min, permeabilized in 0.1% Triton X-100 for 10 min and incubated 1 h with the anti-MRP1 MRPm6 antibody. After washing with the blocking solution for 10 min, cells were incubated with Alexa Fluor 488 anti-mouse IgG fragment for 1 h. Coverslips were mounted on the slides with one drop of antifade reagent (Calbiochem). Localizations of MRP1 and GM1 in the cells were determined with a laser scanning confocal microscope (MRC 1000; Bio-Rad, Hercules, CA) equipped with an argon–krypton laser and Comos software (Bio-Rad).

Purification and reconstitution of MRP1. Purification and reconstitution of MRP1 were carried out as described previously [9].

ATPase activity measurement. The ATPase activity of proteoliposomes was measured as described previously [9].

Transport assays. Standard transport assays were carried out as described previously [13].

Results and discussion

Is MRP1 associated to lipid raft-like structures?

MRP1 and Pgp have been shown to transport the same substrates and their membrane topologies, if we except the amino-terminal end of MRP1, are quite similar. These features would suggest the insertion of MRP1 into DRMs. Western blot analysis of cell-extracted DRMs and confocal microscopy measurements were carried out to determine the localization of MRP1 in the plasma membrane.

Triton X-100 extraction of DRMs

A classical way to demonstrate the insertion of a protein in DRM structures is to isolate them from cells by a cold nonionic detergent. DRMs were extracted from HEK-293 cells overexpressing MRP1 by cold Triton X-100. The extracts were separated on a sucrose density gradient, which was fractionated after ultracentrifugation. The distribution of MRP1 and Flotillin-1, a DRM marker protein [11], among the different fractions was analysed by Western blotting. MRP1 was not associated with the low-density fractions containing Flotillin-1 (fractions 9–12) (Fig. 1) but was detected in fractions 1–6 representative of the Triton X-100-solubilized proteins. Some Flotillin-1 was also detected in these fractions indicating partial solubilization of some DRMs. DRMs migrate at the 30–5% sucrose interface of the gradient (Flotillin-1 was detected in fractions 9–12). No MRP1 was detectable in these fractions suggesting that MRP1 is not localized in DRMs.

Confocal fluorescence microscopy

The localization of MRP1 in the plasma membrane was investigated by confocal fluorescence microscopy on HEK-293-MRP1 cells. DRMs were detected by cholera toxin

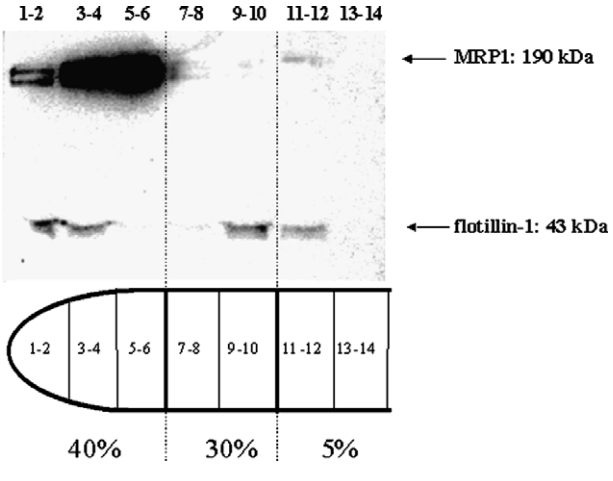

Fig. 1. Sucrose density gradient partitioning MRP1 and marker of classical lipid rafts (Flotillin-1). HEK-293 cells were lysed in 1% (v/v) Triton X-100 at 4 °C and lysates were fractionated by ultracentrifugation on a discontinuous sucrose gradient. A total of 14 fractions were collected and an aliquot of each was run on SDS–PAGE. Proteins were transferred to a nitrocellulose membrane and MRP1 and Flotillin-1 were detected by Western immunoblot analysis.