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Short Communication

LOCALISATION OF THE MULTIDRUG RESISTANCE-ASSOCIATED PROTEIN, MRP, IN RESISTANT LARGE-CELL LUNG TUMOUR CELLS

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Abstract—The drug transport protein, P-glycoprotein, confers multidrug resistance (MDR) by expelling drugs across the cell surface. The structurally similar multidrug resistance-associated protein, or MRP, is also involved with drug efflux. In MDR variants of the human lung tumour cell line COR-L23 that overexpress MRP, there are also changes in intracellular drug distribution. To ascertain whether MRP could be involved in either process, experiments were performed to identify where MRP was located in these cells. Following separation of membranes by sucrose gradient centrifugation, MRP was found predominantly in the lighter membrane fractions containing plasma membrane enzyme activity. Immunofluorescent staining with a monoclonal antibody raised against MRP confirmed that MRP is present at the cell surface of these MDR lung tumour cells.

Key words: multidrug resistance-associated protein; human large-cell lung tumour cells; subcellular localisation; membrane fractionation; immunofluorescent staining; confocal microscopy

The drug efflux protein, P-glycoprotein, encoded by the MDR1 gene, has been implicated in the expulsion of many structurally and functionally unrelated drugs from tumour cells, thus conferring the MDR‖ phenotype [1]. Another protein, MRP, has been identified in a number of *in vitro* selected human tumour cell lines that do not express P-glycoprotein, but show decreased drug accumulation. The gene for MRP overexpressed in the MDR small cell lung tumour line, H69AR has been sequenced [2], and encodes a protein of 1531 amino acids, structurally similar to many ATP binding transmembrane transport proteins. Expression of this gene confers the MDR phenotype [3]. Detected originally as a 190k protein in the MDR human leukemia cell line, HL60Adr [4, 5], MRP has subsequently been found in a number of other non-P-glycoprotein containing MDR cell lines including variants of the large cell lung tumour line COR-L23, and lung adenocarcinoma, MOR [6, 7].

The majority though not all [3] of the cell lines that overexpress MRP show reduced drug accumulation. Changes in intracellular drug distribution have also been observed with fluorescent drug confined to perinuclear vesicles and excluded from the nucleus. This has been described in MDR variants of the human large-cell lung tumour COR-L23 cell line [8, 9]. The role of MRP in this drug redistribution is unclear. It was earlier suggested in work on the HL60/Adr cells that the 190kD protein was located within cells [4, 5], and thus might be directly involved in concentrating drug into vesicles. However, preliminary results of subcellular membrane fractionation studies on the MDR variants of the COR-L23 cell line indicated that the protein was more likely to be at the cell surface [10] in these cells. Since this work was initiated, monoclonal antibodies able to detect MRP by immunocytochemical means have become available, and these show positive staining of the cell surface in cytospin preparations of several MDR cell lines overexpressing MRP [11, 12]. We present here evidence from the subcellular fractionation and from confocal immunocytochemistry undertaken with one of the available monoclonal antibodies showing that MRP resides predominantly at the cell surface of COR-L23/R cells.

Materials and Methods

Cell culture. The MDR variants used were derived from the large-cell (COR-L23) lung tumour cell line by doxorubicin selection as described previously [7]. Cells were grown as attached monolayers in RPMI-1640 medium containing 10% fetal calf serum supplemented with penicillin/streptomycin at 100 IU/mL and 100 μg/mL, respectively. MDR variants were maintained in medium containing doxorubicin at 0.2 μg/mL (COR-L23/R) or 1.0 μg/mL (COR-L23/5010).

Membrane protein fractionation, separation, and analysis. Following cell lysis and a preliminary centrifugation step to remove nuclei and unbroken cells, membranes were prepared as described previously [7]. They were then fractionated by being layered onto a discontinuous gradient of 55%, 40%, 35%, 32%, 29%, and 20% sucrose in lysis buffer, and centrifuged at 150,000 g for 170 min. Material at each interface was collected into the lysis buffer and repelleted. Recovery of protein from the gradient was between 70–80% of that applied. To determine the distribution of MRP in the different fractions, membrane proteins were subjected to western blot analysis using either the affinity purified polyclonal antibody CRA-1, raised against synthetic peptide sequence GTQLSGGQKQRIAIA as outlined previously [7], or the more recently derived antiserum G4, raised in a similar way against peptide sequence GVNLSGGQKQRVSLA (peptides kindly supplied by Dr. W.J. Gullick). Proteins recognised by the antibodies were visualised using an ECL detection system (Amersham International, U.K.). To identify the composition of the different membrane fractions, each fraction was assayed for galactosyl transferase [13] and

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‖ Abbreviations: MRP, multidrug resistance-associated protein; MDR, multidrug resistant; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulphate; TBS, Tris buffered saline.

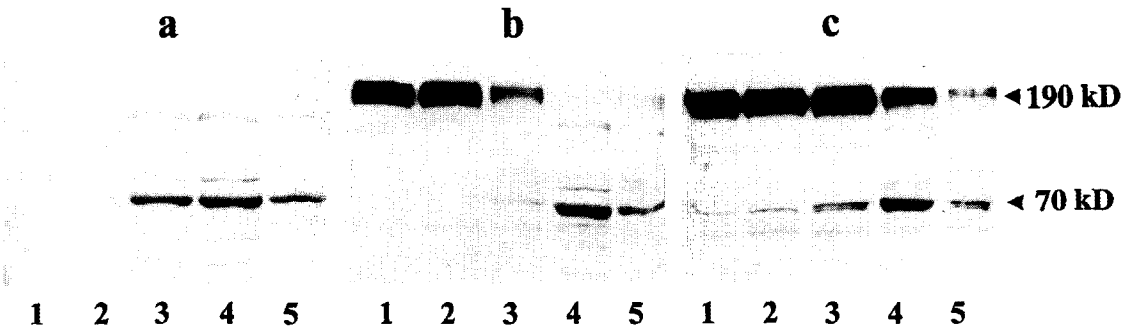

Fig. 1. Western blot analysis of MRP in membrane fractions 1-5 prepared from (a) COR-L23/P cells, (b) COR-L23/R cells, and (c) COR-L23/5010 cells. 20 μg of protein were loaded in each track and filters were probed with polyclonal antiserum, G4.

5'nucleotidase activities [14] by radioenzymatic methods, and for β-galactosidase [13] and glucose-6-phosphatase [15] activities by spectrophotometric methods, and compared with activities in the original membrane preparations prior to fractionation.

Immunocytochemistry. Cells grown on glass slides were washed in PBS, fixed in acetone for 10 min, and frozen down until needed. After thawing, cells were blocked with 1% BSA/Tris buffered saline (TBS), then exposed to the anti-MRP rat monoclonal antibody MRPr1 [11] at 1/1000 dilution in 1%BSA/TBS, followed by biotinylated anti-mouse, -rabbit, and -rat IgG (Sigma Chemical Co., Poole, U.K.) at 1/20 dilution, and finally by fluorescein-streptavidin before mounting and viewing under the confocal microscope. To check that the antibody could gain access to its epitope, in some studies, cells were further permeabilised with 1% Triton X-100 before the blocking step, or 0.5% saponin was included in all the staining solutions. In addition, cells were also tested with the mouse monoclonal antibody, clone no. AC-74, against the cytoplasmic protein β-actin (Sigma Chemical Co., Poole, U.K.).

Results and Discussion

The distribution of MRP in the membrane fractions resulting from sucrose gradient centrifugation was assessed by western

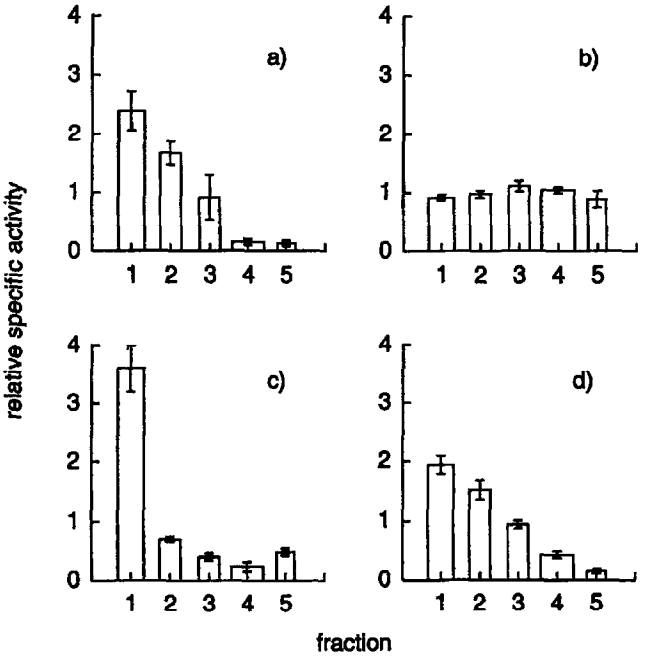

Fig. 2. Distribution of activities of (a) plasma membrane enzyme, 5'nucleotidase, (b) endoplasmic reticulum enzyme, glucose-6-phosphatase, (c) Golgi enzyme, galactosyl transferase, and (d) lysosomal enzyme, β-galactosidase in membrane fractions of COR-L23/R cells following sucrose gradient centrifugation. Values are the mean ± S.E.R of results from four or five separate preparations. Relative specific activities were calculated from the proportion of total enzyme activity in each fraction/proportion of total protein in each fraction. Values over 1 represent enrichment, and values below 1 depletion of activity in a particular fraction. The width of each column represents the proportion of the total protein in that fraction (i.e. 0.22, 0.18, 0.18, 0.28, 0.16 for fractions 1, 2, 3, 4, and 5, respectively); the area of each column represents the proportion of total enzyme activity. Specific activities of each enzyme, calculated as total activity/total protein, ranged between 3–4 nmoles/mg/min for galactosyl transferase, 10–20 nmoles/mg/min for β-galactosidase and for glucose-6-phosphatase, and 10–15 μmoles/mg/h for 5'nucleotidase.

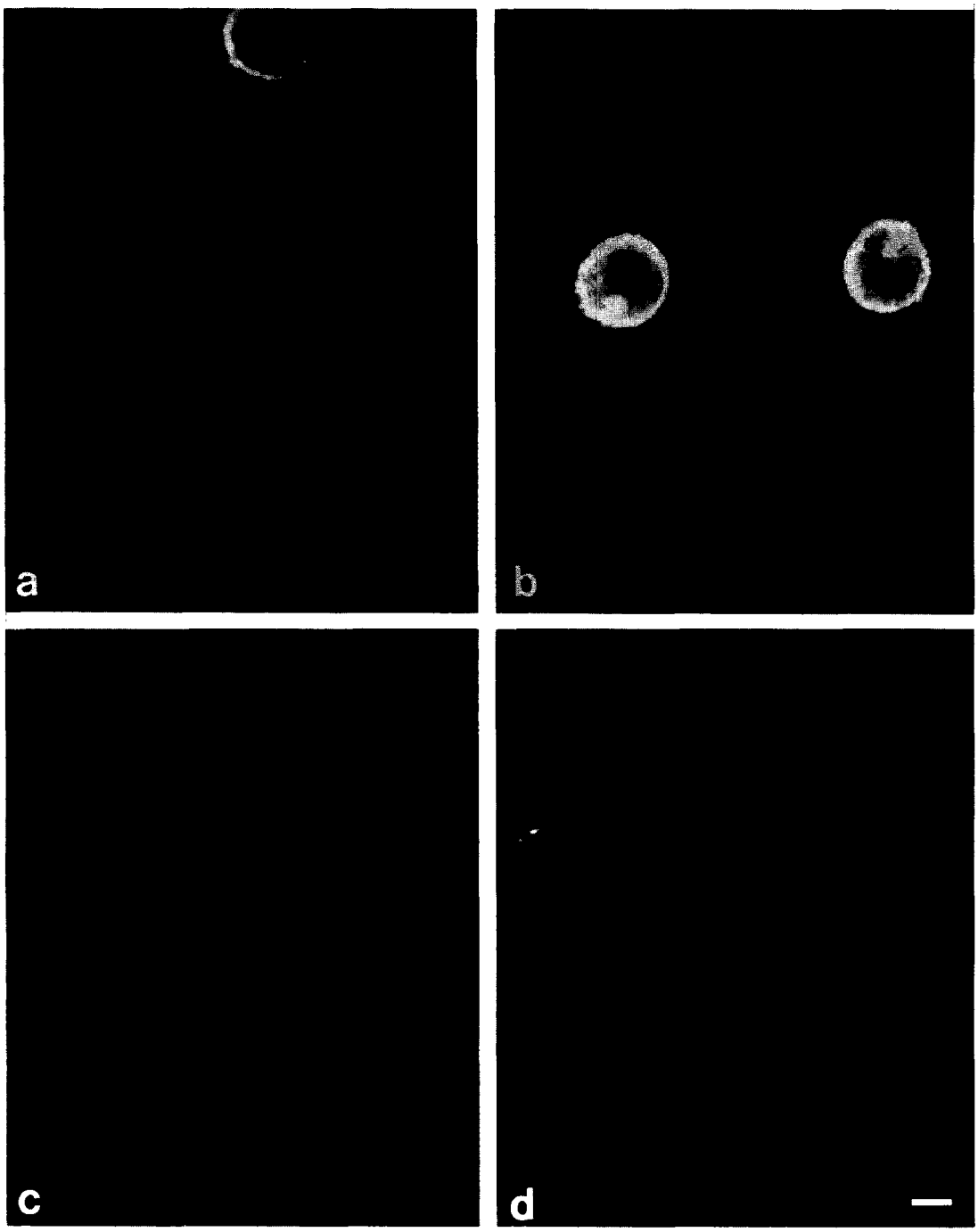

Fig. 3. Immunofluorescent staining with anti-MRP antibody, MRPr1 of (a) COR-L23/R cells, (b) COR-L23/5010 cell, and (c) COR-L23/P cells. Control staining with primary antibody omitted of (d) COR-L23/R cells. Bar = 10 μm.

blot analysis, and the band corresponding to a molecular weight of 190kD taken to be MRP (Fig. 1a–c). Levels of MRP in COR-L23/R membranes were enriched in the three lighter fractions as reported previously [7], and little or none could be detected in the denser membrane fractions 4 and 5. There was more MRP in membranes from the more resistant COR-L23/5010 cells (Fig. 1c), and this was distributed into the heavier fractions as well. Blotted under the same conditions, MRP could not be detected in membranes from the parent cells (Fig. 1a), but was visible in fractions 1 and 2 if blotted using less dilute antibody solutions. In each of the cell types, there was a band in fractions 4 and 5 corresponding to a molecular weight of about 70–75kD, detectable with both polyclonal antibodies. It is unclear whether this is a degradative product of the 190k protein or some other protein that shares homology at the nucleotide binding domain. Inhibition of glycosylation by treat-

ment for 2–3 days with 2 μg/mL tunicamycin led to the appearance of a smaller protein species, 130–160kD in size, in addition to the 190kD protein. Changes in molecular size of MRP following blockade of N-glycosylation have been reported previously by others [5, 16].

The subcellular origins of the membranes contained in each fraction were established by measuring activities of certain marker enzymes. Results shown are from the COR-L23/R line (Fig. 2), and similar results were obtained in the other two cell lines. As can be judged by the distribution of the activities of the Golgi enzyme, galactosyl transferase (Fig. 2c) and the microsomal marker enzyme, glucose-6-phosphatase (Fig. 2b), Golgi vesicles were enriched in fraction 1 only whilst microsomal membranes were fairly evenly distributed throughout the five fractions. Activities of both plasma membrane enzymes, 5'nucleotidase (Fig. 2a), and lysosomal enzyme β-galactosidase, (Fig. 2d) appeared to be enriched in fractions 1 and 2 with some present in fraction 3, a distribution very similar to that of the MRP detected immunologically in those fractions. When western blot filters were probed with streptavidin-linked alkaline phosphatase, bands of biotin-containing mitochondrial proteins could be detected. These bands were predominantly in fractions 4 and 5. MRP distribution was thus most similar to that of the plasma membrane enzyme, though a possible association with lysosomes could not be ruled out.

To confirm the presence of MRP at the cell surface, cells grown on glass slides were permeabilised by fixation and subjected to immunofluorescent staining with the anti-MRP monoclonal antibody, MRPr1 [11]. Under the confocal microscope, a strong band of intense fluorescence could be seen around the outer surface of the MRP-containing cells COR-L23/R (Fig. 3a) and COR-L23/5010 (Fig. 3b). A small amount of cytoplasmic fluorescence was seen in these cells and also in the parent cells, COR-L23/P (Fig. 3c) and in the controls exposed only to fluorescein-streptavidin without the initial primary antibody step (Fig. 3d). This staining most probably represents endogenous biotin covalently linked to mitochondrial proteins. No additional staining was observed inside the cells following extra permeabilisation with Triton X-100 or 0.5% saponin. Indeed, there appeared to be good access for antibodies to the cell interior, cytoplasmic staining being obtained using a monoclonal antibody against β-actin (results not shown).

A cell surface location for MRP has been reported by others in certain drug-selected MRP-expressing MDR lung tumour cell lines [11, 16] and in MRP transfected HeLa cells [16]. The intracellular location reported in the human leukemic line HL60/Adr [4, 5] may reflect differences in cell type or in MRP sequence in these cells [16]. Since MRP appears to be located primarily at the plasma membrane in our COR-L23/R cells with only a small amount associated with the intracellular perinuclear vesicles, it is unlikely that traffiking to the cell surface of drug sequestered into these vesicles as observed under the confocal microscope [8] could provide the major means of drug efflux. Similar drug redistribution into vesicles has been seen in P-glycoprotein-containing cells [17], where extensive efflux across the plasma membrane is known to occur. The MRP staining within the COR-L23/R cells did not correspond closely with the perinuclear drug distribution observed previously in these cells [8]. It is thus not clear whether MRP is responsible directly for such drug redistribution. Indeed, how MRP at the cell surface brings about drug efflux from COR-L23/R cells is as yet unclear, though glutathione is known to be important [18]. In some cells, MRP has been shown to transport glutathione S-conjugates [19], but there is no direct evidence that the drugs themselves are transported out in conjugated form.

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