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SORCIN (V19), A SOLUBLE ACIDIC CALCIUM-BINDING PROTEIN OVERPRODUCED IN MULTIDRUG-RESISTANT CELLS

IDENTIFICATION OF THE PROTEIN BY ANTI-SORCIN ANTIBODY

MARIAN B. MEYERS,* KATHERINE A. SCHNEIDER, BARBARA A. SPENGLER, TIEN-DING CHANG and JUNE L. BIEDLER

Laboratory of Cellular and Biochemical Genetics, Memorial Sloan-Kettering Cancer Center, New York, NY 10021, U.S.A.

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Abstract—Sorcin (soluble resistance-related calcium-binding protein), an acidic (pI = 5.7) protein ($M_r \sim 20$ kDa) previously designated V19, was originally identified in cells selected for high levels of resistance to vincristine. Two-dimensional gel electrophoresis and/or Western blot techniques now show sorcin to be overproduced in cells selected for resistance to actinomycin D (QUA/ADj), colchicine (CH^RC5), and adriamycin (BE(2)-C/ADR). Not all cell lines selected for resistance to these drugs overproduced sorcin; e.g. cells of an independently selected actinomycin D-resistant subline of QUA, QUA/ADsx, did not contain increased amounts of sorcin. Sorcin was purified by preparative gel electrophoresis from QUA/ADj cells and used to generate specific antiserum in chickens. By Western blot analyses the antiserum was shown to recognize sorcin in QUA/ADj and in vincristine-resistant mouse and Chinese hamster lung, colchicine-resistant Chinese hamster ovary, and adriamycin-resistant human neuroblastoma lines. Low level expression of the protein was detectable in control, drug-sensitive cells. Direct binding assays with $^{45}Ca^{2+}$ showed that sorcin was a calcium-binding protein. QUA/ADj cells contained increased numbers of double minute chromosomes (DMs), cytogenetic indicators of gene amplification. As found for two other multidrug-resistant sublines, sorcin overproduction in QUA/ADj cells may be the result of amplification of the sorcin-encoding gene. The overproduction of this protein in multidrug-resistant cells of various species implies that sorcin plays a role in expression of the resistant phenotype.

The use of chemotherapeutic agents to treat cancer is limited frequently by the development of drug-resistant tumor cell populations. One approach to this problem is through experimental derivation and analysis of biochemical changes in cells selected *in vitro* for acquired resistance to drug. Cells selected for survival in drugs such as vincristine or actinomycin D are termed multidrug-resistant because they are not only resistant to the selective agent but also cross-resistant to a variety of other natural products or semi-synthetic analogs used in the treatment of cancer [1, 2]. This laboratory has described a number of concomitants of multidrug resistance in hamster, mouse, and human sublines including overproduction of a plasma membrane glycoprotein, gp150-180 [3, 4], also identified in other laboratories [5–7], and a soluble acidic protein, sorcin (V19) [8, 9]. Multidrug-resistant cell lines developed in other laboratories also may contain increased amounts of proteins with molecular weights at or near 20 kDa [10–14]. Sorcin was first identified in vincristine-resistant cells and was not found in independently-selected actinomycin D- and daunorubicin-resistant Chinese hamster sublines [8]. However, more recent examination of a wider variety of cells revealed the presence of sorcin in an actinomycin D-resistant mouse tumor cell designated QUA/ADj [2]. Association of sorcin with multidrug resistance rather than exclusively with vincristine resistance thus became a possibility.

Sorcin protein isolated from QUA/ADj cells by preparative gel electrophoresis was used to generate antiserum in chickens. By Western blot techniques, the antibody was found to recognize sorcin from a number of other cell lines. The presence of four putative calcium binding sites in the deduced amino acid sequence of sorcin [15], together with the report from another laboratory [13] of calcium-binding properties of an acidic 22 kDa protein overproduced in multidrug-resistant cells, led to the use of direct binding $^{45}Ca^{2+}$ assays to show that sorcin is a calcium-binding protein. Of importance in this study is the finding that QUA/ADj cells, like all sorcin-overproducing lines, display obvious cytogenetic manifestations of gene amplification [8, 9, and this report]. Gene amplification has been shown to be the mechanism for increased synthesis of gp150-180 (or P-glycoprotein) in a number of multidrug-resistant sublines [16–19]. The gene encoding sorcin is co-amplified with the P-glycoprotein gene in two sorcin-overproducing resistant lines [15, 18, 20, 21] derived from Chinese hamster cells. Increased synthesis of

* To whom reprint requests should be addressed at: Sloan-Kettering Institute, 145 Boston Post Road, Rye, NY 10580.

sorcin may be the result of amplification of the sorcin-encoding gene in QUA/ADj cells as well.

MATERIALS AND METHODS

Materials. Radioactive chemicals were purchased from New England Nuclear (Boston, MA) and other chemicals from the Sigma Chemical Co. (St. Louis, MO). Vincristine was a gift from Eli Lilly. Supplies for gel electrophoresis were Bio-Rad (Rockville Centre, NY) products. The secondary antibodies were purchased from Cooper Biomedical, Inc. (Malvern, PA); sonication equipment was from Sonics & Materials, Inc. (Danbury, CT), sample concentrator for protein electroelution from ISCO, Inc. (Lincoln, NE), and densitometer from Hoefer Scientific Instruments (San Francisco, CA).

Cells and cell culture. QUA cells were cloned from a line established in cell culture from a 3-methylcholanthrene-induced tumor of a C57BL/6 mouse [2]. Drug-resistant QUA/ADj and QUA/ADsx lines were selected with actinomycin D by stepwise 2-fold increases in drug concentration. The sublines were maintained as monolayer cultures in 5.0 and 1.0 μg/ml of drug, respectively, in a 1:1 mixture of Eagle's Minimum Essential Medium and Ham's F12 supplemented with 5% fetal bovine serum. The Chinese hamster ovary line AUXB1 and the colchicine-resistant subline CH^RC5 were provided by Dr. Victor Ling [22]. Other cell lines used in this study, DC-3F/VCRd-5L and DC-3F/AD X (multi drug-resistant sublines of DC-3F, a Chinese hamster lung cell line) and MAZ/VCR (a vincristine-resistant subline of MAZ, a mouse tumor line cloned from the same cell population as QUA), have been described [8, 9]. The BE(2)-C/ADR subline was selected with adriamycin from BE(2)-C, a cloned human neuroblastoma line [23], by stepwise 2-fold increases in drug concentration; cells are currently maintained as monolayer cultures in 2.0 μg/ml of adriamycin. Drug sensitivity was measured in a 6-day growth assay as previously described [1]. Metabolic labeling of cells for two-dimensional gel electrophoresis was carried out by replacing growth medium with methionine-free medium plus standard serum and 10 μCi/ml [^{35}S]methionine for 4 hr before harvest.

Isolation of sorcin, production of antiserum, and gel electrophoresis. Two-dimensional gel electrophoretic procedures are those of O'Farrell *et al.* [24] as used in this laboratory [4]. The second dimension gels [25] were 10–13% acrylamide gradients with dimensions of 0.075 × 14 × 30 cm. Preparative gel electrophoresis procedures described by LeStourgeon and Beyer [26] were used to purify sorcin. QUA/ADj cells were lysed by sonication (3 × 10 sec pulses with microtip) in 2 ml of 20 mM sodium phosphate buffer (pH 6.8). Aliquots (1 ml) of 13,000 *g* supernatant fractions (5–10 mg protein) were applied to preparative 12% acrylamide gels (0.3 × 14 × 11 cm) containing a flow-through port. Fractions (5 ml) of eluant were collected, concentrated, and analyzed by gradient gel electrophoresis and Coomassie Blue staining for sorcin content. Total QUA/ADj protein was used as a marker to identify the sorcin position. Acrylamide slices containing sorcin were excised and injected subcutaneously into chickens by the Pocono Rabbit Farm and Laboratory of Canadensis, PA. Each animal was injected three times over the course of 4 months with about 60 μg of protein per injection. After a 6-month interval, animals were boosted with an additional 60 μg of protein electroeluted from the acrylamide slices into a pH 8 buffer containing 4.8 mM Tris and 189 mM glycine. Eluted protein was measured as described by Lowry *et al.* [27] for estimation of the amount of sorcin in QUA/ADj cells. Antibody was harvested from egg yolks according to published procedures [28].

Western transfer procedure. Cells were lysed in 1% Triton X-100 in 50 mM Tris (pH 7.2). Aliquots of 20,000 *g* supernatant fractions containing 10–300 μg of protein were electrophoresed on 12% acrylamide gels (0.075 × 14 × 11 cm). The completed gels were transferred onto nitrocellulose according to procedures described by Towbin *et al.* [29]. Sheets were soaked at room temperature in 5% powdered milk in 0.1 M Tris (pH 7.4) followed by a 16-hr incubation at 4° in a 1:100 dilution of anti-sorcin antibody (1 mg/ml) or anti-gp150-180 rabbit antibody [30] in 5% milk. The sheets were washed five times in 0.1 M Tris (pH 7.4) and then incubated for 1 hr at room temperature in a 1:500 dilution of peroxidase-conjugated rabbit anti-chicken or goat anti-rabbit antibody. After washing, the sheets were placed in substrate solution consisting of 4 ml of 4-chloro-1-napthol in methanol (3 mg/ml), 20 ml of 15 mM NaCl in 50 mM Tris (pH 7.4), and 12 μl of 30% hydrogen peroxide for color development.

Direct calcium binding assay. Calcium binding procedures described by Maruyama *et al.* [31] were used. Cells were lysed by sonication in 10 mM imidazole containing 1 mM EDTA (pH 7.4), and 100–150 μg of protein from low speed supernatant fractions was subjected to electrophoresis on 12% polyacrylamide gels [25]. Proteins were transferred to nitrocellulose [29]. The sheets were washed, incubated with $^{45}Ca^{2+}$, and prepared for radioautography as reported [31]. After radioautography, sheets were treated with anti-sorcin antibody and developed as described above for further identification of the calcium-binding protein as sorcin.

RESULTS

Characterization of QUA, QUA/ADj, and QUA/ADsx cell lines. Tumor-derived QUA cells manifest growth characteristics of malignant cells in culture. They grow as a loose, disordered network of fibroblast-like cells which, as the culture ages, aggregate to form large clusters loosely attached to the substrate (Fig. 1a). By contrast, resistant sublines exhibit the reverse transformation phenotype seen also in multidrug-resistant Chinese hamster and the vincristine-resistant MAZ (MAZ/VCR) sublines [2, 32]. Cells of the QUA/ADj cell line are flattened fibroblast-like cells which orient to form swirling arrays of contiguous cells (Fig. 1b), whereas QUA/ADsx cells have an epithelioid to stellate morphology and form a contiguous monolayer with no focal mounding (Fig. 1c). The normalization or reverse transformation of the resistant cells is further

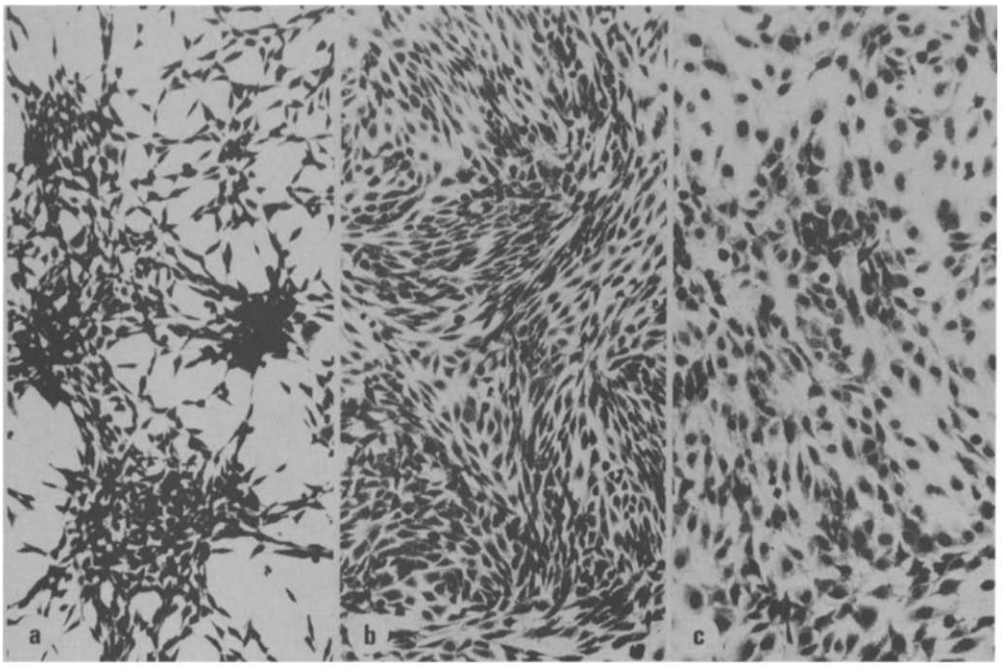

Fig. 1. Monolayer cultures of QUA (a), QUA/ADj (b), and QUA/ADsx (c) stained with May-Grunwald Giemsa. Magnification: 75×.

manifested in the reduced tumorigenic potential of these cells, as determined in either a syngeneic or heterotransplantation assay and discussed extensively elsewhere [2]. QUA/ADj and QUA/ADsx were cross-resistant to vincristine and colchicine (Table 1), characteristic of multidrug resistance.

QUA and QUA/ADsx cells both contain approximately 15 double minute chromosomes (DMs) per cell; QUA/ADj cells contain 7-fold more, an average of 106 DMs per cell. MAZ/VCR cells also contain increased numbers of DMs (compared to MAZ), the presence of which has been correlated with amplification of genes coding for the resistance-related surface glycoprotein gp150-180 [19]. Overproduction of gp150-180 was detected in QUA/ADj and QUA/ADsx cells by Western blot analysis with polyclonal antibody to gp150-180 (Fig. 2 and data not shown). All other drug-resistant lines described in this report contained increased amounts of this plasma membrane component compared to controls ([4, 30] and unpublished results). The precise role of this glycoprotein is unknown; one hypothesis associates it with drug transport [16]. QUA/ADj and QUA/ADsx, like other multidrug-resistant cells [1, 33], accumulate less intracellular drug than control cells. Uptake of tritiated actinomycin D by QUA/ADj and QUA/ADsx is decreased to 1.4 and 5.5%, respectively, of control levels [2, 34]. Gp150-180 may control this decreased accumulation. The identity of the genes contained in the DMs characterizing parental QUA (and MAZ) cells is unknown, although in light of the malignant character of these lines the DMs may comprise amplified oncogenes.

Identification and purification of sorcin for antibody production. Sorcin was detected in QUA/ADj cells on autoradiograms of two-dimensional gels of [^{35}S]methionine-labeled soluble proteins (Fig. 3). The spot indicated by an arrow on the QUA/ADj gel shown in Fig. 3b had the same apparent molecular weight and pI as sorcin in vincristine-resistant mouse and Chinese hamster lung cells [8, 9]. The sorcin spot was not visible on QUA or QUA/ADsx cells at this exposure (Fig. 3, a and c). Figure 4 depicts a Coomassie-stained gel of proteins in selected frac-

Table 1. Characteristics of QUA, QUA/ADj, and QUA/ADsx cells

Cell line	Resistance and cross-resistance to: Actinomycin D		Vincristine		Colchicine		Sorcin	Gp150-180
	EC_{50}*	Increase	EC_{50}	Increase	EC_{50}	Increase		
QUA	0.0031	1	0.014	1	0.0089	1	–	–
QUA/ADj	2.9	9355	4.3	307	8.8	989	+	+
QUA/ADsx	0.44	1419	2.2	157	3.9	438	–	+

* EC_{50} values represent concentration of drug which inhibits growth to 50% of control level.

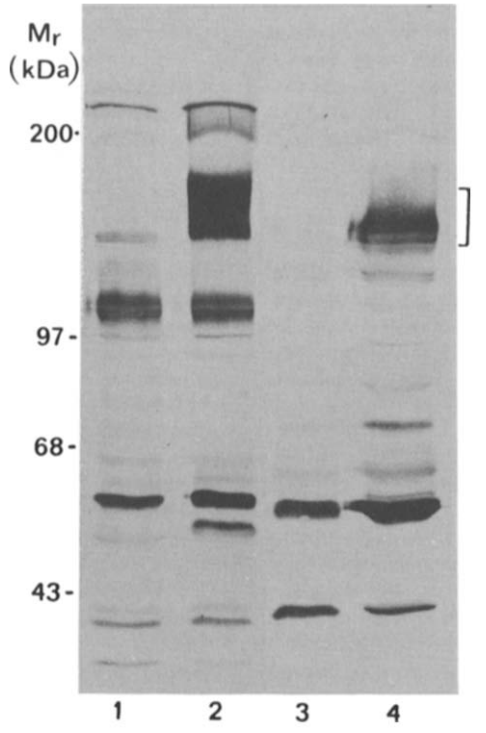

Fig. 2. Western blot analysis of proteins from DC-3F (lane 1), actinomycin D-resistant DC-3F/AD X (lane 2), QUA (lane 3), and QUA/ADj (lane 4) cells with polyclonal antibody to gp150-180. Bracket indicates gp150-180. Aliquots containing 100 μg of protein were analyzed. Antibody was raised against hamster cell gp150-180 [30] and recognizes the corresponding mouse protein.

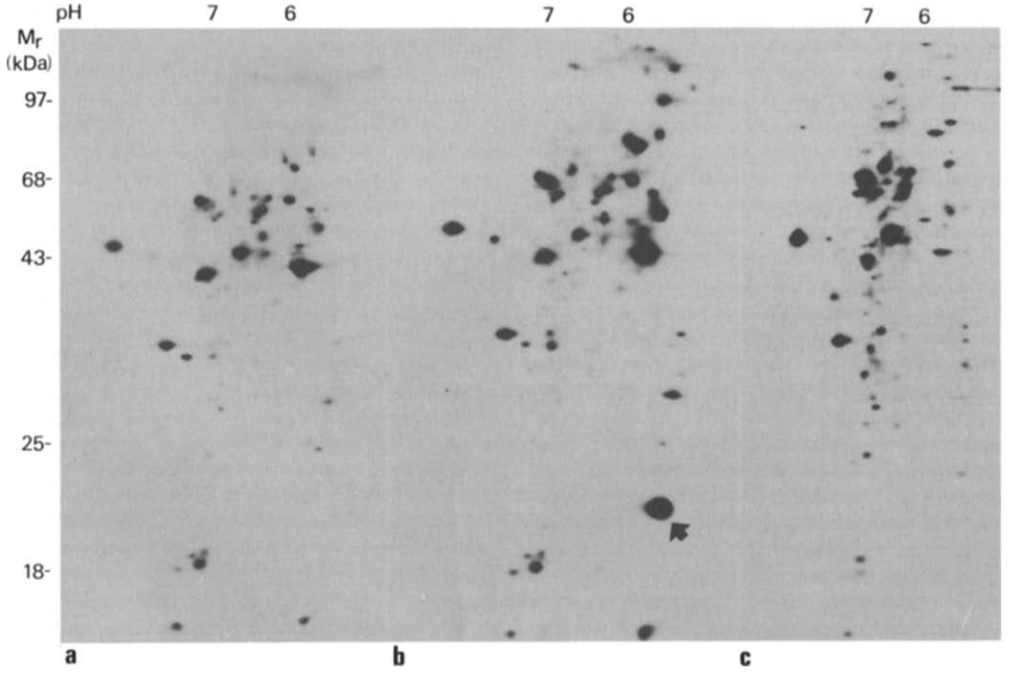

Fig. 3. Autoradiogram of two-dimensional gels of [^{35}S]methionine-labeled soluble proteins in QUA (a), QUA/ADj (b), and QUA/ADsx (c) cells. Aliquots containing 1×10^6 cpm were examined. Dried gels were exposed to Kodak X-OMAT X-ray film at −70° for 1 week. The arrow indicates sorcin.

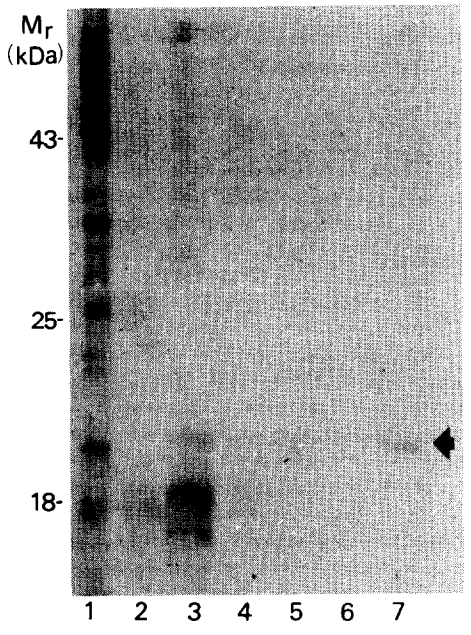

Fig. 4. Coomassie-stained gel displaying total soluble proteins in QUA/ADj cells (lane 1) and fractions separated by preparative gel electrophoresis (lanes 2–7). Sorcin is indicated by an arrow. The protein was the major component in the fraction in lane 7.

tions from a preparative gel electrophoretic separation of QUA/ADj soluble proteins. The single band in lane 7 was sorcin. Bands from at least two such gels were excised, pooled, and used as the source of antigen for immunization (see Materials and Methods).

Use of anti-sorcin antibody to determine the presence of sorcin among multidrug-resistant cell lines. Western transfers with sorcin antibody from immune chicken sera or egg yolks demonstrated that sorcin was overexpressed in QUA/ADj (Fig. 5, lane 5), as well as in DC-3F/VCRd-5L, MAZ/VCR, and CH^RC5 (Fig. 5, lanes 2, 7, and 9) and BE(2)-C/ADR cells (Fig. 6, lane 6). The presence of sorcin could be detected in control QUA, DC-3F, and MAZ cells; however, visualization of sorcin in these sublines required examination of at least 300 μg of total soluble protein. The resulting sorcin bands were too pale to be photographed but could be quantitated by a densitometer. Analysis of densitometric tracings showed that DC-3F/VCRd-5L cells contained 15- to 30-fold more sorcin than DC-3F cells, and QUA/ADj cells contained 50- to 60-fold more sorcin than QUA cells. Approximately 1% of total soluble protein in QUA/ADj cells was sorcin as determined by measurement of total protein applied to preparative gels and of sorcin eluted from second step polyacrylamide gels (Fig. 4). The antiserum also recognized sorcin from MC-IXC/VCR, a vincristine-resistant human neuroblastoma line (data not shown). The identity of the additional bands in lanes 8 and 9 of Fig. 5 is not known.

Direct $^{45}Ca^{2+}$ binding assay. Sorcin is a calcium-binding protein as shown by direct $^{45}Ca^{2+}$-binding assays (Fig. 6). It was a major calcium-binding protein in QUA/ADj cell cytosol (Fig. 6, lane 2, upper arrow). No corresponding band was observed among QUA cell proteins (Fig. 6, lane 1). Human sorcin also binds calcium as shown in Fig. 6, lane 4 (lower arrow). The identification of the human calcium-binding protein as sorcin was confirmed with the use of the anti-sorcin antibody (Fig. 6, lane 6). The cross-reacting human protein migrated slightly faster on gels than rodent sorcin, suggestive of a lower molecular weight for human sorcin. Sorcin is also a major calcium-binding protein in DC-3F/VCRd-5L cells (data not shown), which synthesized increased amounts of sorcin (Fig. 5).

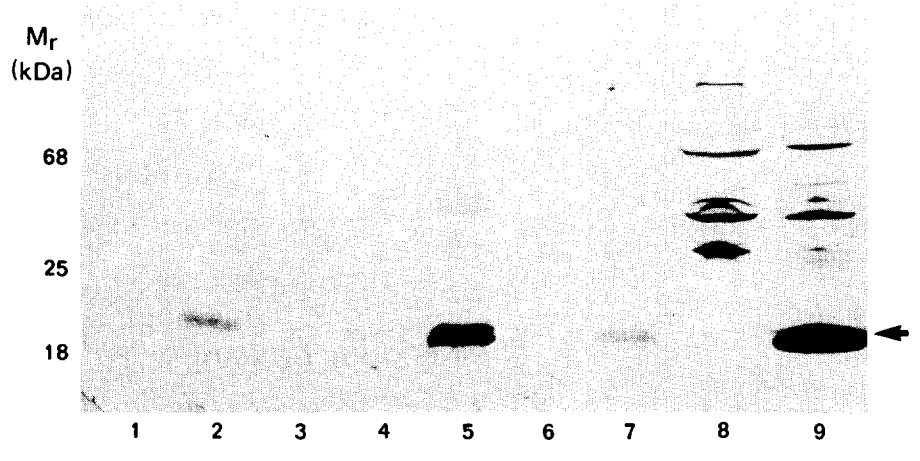

Fig. 5. Western blot analysis with sorcin antibody of proteins in DC-3F (lane 1), DC-3F/VCRd-5L (a vincristine-resistant HSR-containing DC-3F subline) (lane 2), DC-3F/AD X (lane 3), QUA (lane 4), QUA/ADj (lane 5), MAZ (lane 6), MAZ/VCR (vincristine-resistant subline of MAZ containing large numbers of DMs) (lane 7), AUXB1 (lane 8), and CH^RC5 (colchicine-resistant HSR-containing subline of AUXB1) (lane 9). One hundred micrograms of protein from each line was examined. The arrow indicates sorcin.

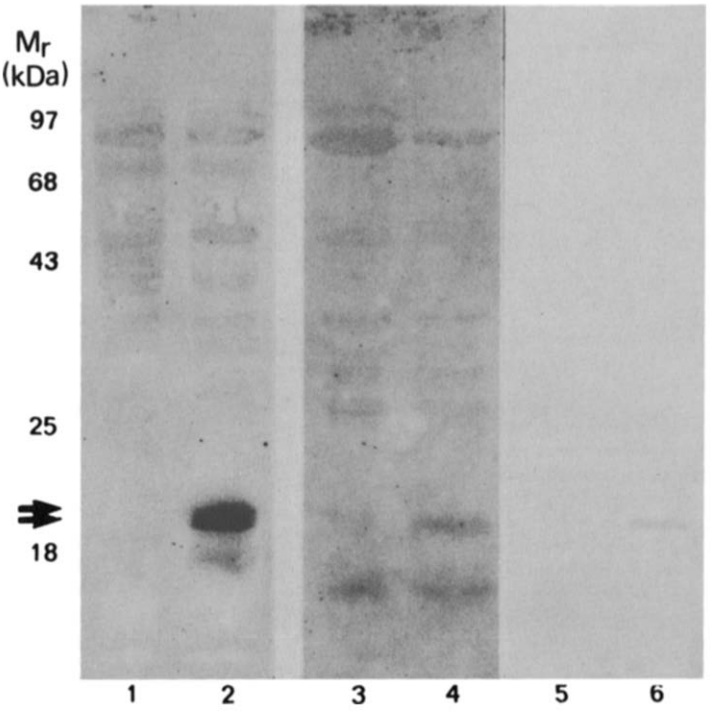

Fig. 6. Demonstration of the calcium-binding property of sorcin. Autoradiogram of direct $^{45}Ca^{2+}$-binding to proteins in QUA (lane 1), QUA/ADj (lane 2), BE(2)-C (lane 3), and BE(2)-C/ADR (60-fold adriamycin resistant subline of BE(2)-C containing large numbers of DMs) (lane 4) and Western analysis of BE(2)-C (lane 5) and BE(2)-C/ADR (lane 6) cytosol proteins with anti-sorcin antibody. Aliquots containing 100 μg of protein were analyzed. Human sorcin migrated slightly faster than rodent sorcin, as indicated by the double arrows.

DISCUSSION

Sorcin was first reported to be overproduced in four independently-selected vincristine-resistant cell lines [8, 9] and is now shown, by two-dimensional gel electrophoresis and/or use of sorcin antibody, to be highly expressed in some cells resistant to actinomycin D, colchicine, and adriamycin as well. The anti-sorcin antibody also recognizes a 22,000-dalton calcium-binding protein (CP_{22}) in adriamycin-resistant mouse and human cells described by another laboratory ([13] and unpublished results).

The spots representing sorcin on two-dimensional gels of proteins from various resistant sublines were heretofore presumed to represent the same protein [8, 9]. Recognition of these peptides by anti-sorcin antibody in the present study indicates a common antigenic site, a confirmation of identity or homology among the sorcin proteins from all lines studied. The basis of the slight difference in migration of human and rodent sorcin is not known. It may be due to a primary structure difference or to post-translational modification. The overproduction of sorcin in cells from three different species selected for resistance to at least four different drugs suggests that sorcin could contribute to the multidrug-resistant phenotype.

Sorcin is a major calcium-binding protein in those cells which overproduce it. Its abundance raises the question of what effect it may have on the physiology of the resistant cell. The protein may normally play a role in regulating intracellular calcium content. As a calcium level modulator, increased sorcin may be associated with the normalized phenotype of multidrug-resistant cells, the nature of which is discussed in this report and elsewhere [2]. Neoplastic cells proliferate at low calcium levels, whereas normal cells have a higher calcium requirement [35]. Speculatively, multidrug-resistant normalized cells may utilize calcium in a different manner than drug-sensitive malignant cells, and sorcin may be part of that altered calcium pattern. Also, because the calcium environment within a cell may be linked to drug transport control in drug-resistant cells [36], sorcin could participate in this aspect of multidrug resistance. It should be noted that increased sorcin amount is not an obligatory concomitant of multidrug resistance. Of eleven resistant sublines established in this laboratory and examined for sorcin, six overproduce the protein. DC-3F/VCRd-5L, DC-3F/VCRm [9], MAZ/VCR, QUA/ADj, MC-IXC/VCR [9] (a vincristine-resistant neuroblastoma line) and BE(2)-C/ADR are overproducers. By contrast, DC-3F/ADX, DC-3F/DMXX (a daunorubicin-resistant subline of DC-3F [2]), MAZ/ADs-4 (an actinomycin D-resistant subline of MAZ [2]), QUA/ADsx, and SH-SY5Y/VCR (vincristine-resistant subline of SH-SY5Y, a human neuroblastoma line) synthesize sorcin at a level comparable to or only slightly greater than that of control cells. As expected, antifolate-

resistant DC-3F cells do not have increased amounts of sorcin [8].

That increased synthesis of sorcin could be the result of amplification of the gene encoding the protein was postulated in earlier studies [9]. This hypothesis was supported by the finding that only the sorcin-overproducing cell lines have obvious gene amplification-associated cytogenetic abnormalities. For example, QUA/ADj, MAZ/VCR, and BE(2)-C/ADR cells have DMs, and DC-3F/VCRd-5L cells have homogeneously staining regions (HSRs) [9 and this report]. Additional support of a gene amplification hypothesis was provided by the observation that sorcin synthesis decreases in MAZ/VCR and DC-3F/VCRd-5L cells when these cells are grown in the absence of drug; the cells revert to relative drug sensitivity in parallel with decline in DM number and HSR length respectively [9]. The speculation was subsequently shown to be accurate in the case of DC-3F/VCRd-5L and CH^RC5 cells; amplification of the sorcin gene was demonstrated by molecular methods [15]. The gene that encodes sorcin is apparently tightly linked and co-amplified with the membrane glycoprotein gp150-180 (or P-glycoprotein) gene in those cells [15, 18, 20, 21]. Increase in copy number of the membrane glycoprotein gene may or may not be accompanied by amplification of the sorcin gene [15, 18, 21]. For example, DC-3F/AD X and DC-3F/DM XX cells amplify gp150-180 (P-glycoprotein) genes but not the sorcin gene [21]. P-glycoprotein is overproduced in these cells whereas sorcin is not. The postulate at this time is that overproduction of both gp150-180 and sorcin in QUA/ADj cells is the result of gene amplification. Whether overproduction of the two proteins is coordinately controlled and whether the proteins are functionally interrelated in these or other resistant cells is an open question. A recent report demonstrates that overexpression of P-glycoprotein is sufficient to confer cellular resistance to vincristine, adriamycin, and colchicine [37], suggesting that sorcin may not be directly involved in establishing the resistant phenotype but, rather, may play a role in modulating its character.

Previous studies have shown that sorcin is phosphorylated [38]. The sorcin antibody will be used to further explore this finding and its relationship to the calcium-binding functions of sorcin. Studies are in progress to localize sorcin by indirect immunofluorescence with the use of the antibody. The function of sorcin in normal cells, as well as a possible role for sorcin in multidrug resistance development, is now being sought.

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