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Association of sorcin with drug resistance in L1210 cells*

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Summary. L1210 sublines independently selected for resistance to teniposide (VM-26), etoposide (VP-16), doxorubicin (DOX), dactinomycin (DACT), or vincristine (VCR) express an anionic, 22-kDa protein that is not observed in extracts of parental L1210 cells. Antibody raised against sorcin, an acidic calcium-binding protein overproduced in many other cells resistant to these agents, cross-reacts with the 22-kDa polypeptide. The levels of the 22-kDa protein (sorcin) increase with the relative levels of drug resistance of the L1210 sublines. The appearance of sorcin in these various sublines further supports the notion that the overproduction of this protein is related to the general phenomenon of multidrug resistance rather than to specific drug resistance and that selection for resistance to teniposide produces L1210 sublines with multidrug resistance.

Introduction

Resistance to teniposide (VM-26) in murine L1210 sublines is accompanied by the coincidental expression of cross-resistance to other agents [23]. Cross-resistance was expressed to vincristine (VCR), for which no common pharmacological action with VM-26 is known, and to a more restricted group of drugs [i.e., etoposide (VP-16), doxorubicin (DOX), dactinomycin (DACT), and amsacrine (mAMSA)] which, like VM-26, commonly stabilize a transitional intermediate of type II DNA topoisomerase (TOPO II) with DNA. The influx of VM-26 was slower for L1210/VM-26 sublines than for parental cells, and the ninefold range in the rate of influx for a spectrum of sublines correlated inversely with the increase in resistance [14]. The efflux of VM-26 was also faster for these sublines than for parental cells. Cellular levels of the drug were lower in these sublines; however, adjustment for equitoxic concentrations of extracellular drug indicated that higher cellular levels of VM-26 were required for the inhibition of L1210 sublines than for that of parental cells. As in other multidrug-resistant sublines, the cellular level of VM-26 was increased by both verapamil and potassium cyanide, although the increase was only two- and sevenfold, respectively, for the 1,300-fold more resistant, prototype L1210/VM-26 subline, LIa5μ*M* [23].

The resistance of L1210/VM-26 sublines was associated with the appearance of chromosomes, generally two, with homogeneously staining regions that were not evident in parental or revertant cells [13]. Although our efforts to associate resistance to VM-26 with the appearance of a resistance-related glycoprotein [8] were initially unsuccessful [23], the technique of immunoblotting with a polyclonal antibody to the multidrug-resistant P-glycoprotein indicated small amounts of two cross-reacting proteins with the electrophoretic mobility of 150- to 155-kDa proteins in LIa5μ*M* cells (Meyers and Roberts, unpublished observation). No similar proteins were detected in plasma membranes from parental cells.

Resistance in these L1210/VM-26 sublines was also associated with the appearance of a 22-kDa protein that was not detected in similar extracts of parental cells [23]. Furthermore, the protein disappeared with the reversion of resistant sublines. The distribution of the 22-kDa protein among subcellular fractions was altered by the addition of calcium ions [23]. Small proteins that range in size from 19 kDa to 30 kDa have also been reported for other sublines [1, 2, 5, 6, 11, 15–19, 24, 26], and in one instance such a protein was identified as a subunit of glutathione transferase [1].

Sorcin, a 22-kDa acidic protein overproduced in vincristine-resistant Chinese hamster lung cells due to amplification of the sorcin gene, is known to be a calcium-binding protein [19, 26]. The question as to whether or not the 22-kDa protein in L1210/VM-26 cells and sorcin are the same or similar was addressed by conducting Western transfer analysis with the antibody to sorcin. In addition, the present study demonstrated that the 22-kDa protein in L1210 sublines selected independently for resistance to VM-26, VP-16, VCR, DOX, or actinomycin D also reacts with the antibody to sorcin.

Materials and methods

Materials. VM-26 and VP-16 were generously donated by Bristol Myers Co., Syracuse, NY, and were dissolved in dimethylsulfoxide. DOX, DACT, and VCR were products of Adria Laboratories, Columbus, Ohio; Merck, Sharp and Dohme, West Point, Pa; and Eli Lilly and Co., Indi-

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anapolis, Ind; respectively. Cells were routinely cultured in AutoPow, an autoclavable formulation of Eagle's minimum essential medium, supplemented with 10% horse serum and L-glutamine from Gibco Laboratories, Grand Island, NY. L-Methionine and phenylmethylsulfonyl fluoride (PMSF) were purchased from Sigma Chemical Co., St. Louis, Mo. Chemicals and equipment for electrophoresis were obtained from BioRad Laboratories, Richmond, Calif. Nonidet P-40 (NP-40) was gratuitously provided by Shell Chemical Co., New York, NY, and L-[^{35}S]-methionine was purchased from New England Nuclear Products, Boston, Mass.

Selection of resistant sublines. The selection of resistant sublines generally started with concentrations of drugs equivalent to fivefold the IC_{50} value for L1210 cells. The drug was added to ten stationary suspension cultures of 10 ml each that were inoculated concomitantly with 300,000 cells/ml from logarithmically growing cultures. Between 7 and 14 days later the few surviving cells, which were not evident by microscopic examination, were collected by centrifugation and resuspended in drug-free medium. These cultures were allowed to grow until repopulation was evident, at which time the original concentration of the drug was again added to the two cultures with the largest number of cells. By this procedure, the population of resistant cells generally increased in several weeks to a level where portions could be expanded for freezing and continuation of the selection. This process was repeated twice, with the concentration of the drug increasing two- to fivefold for each subsequent selection. After the third selection, cells were cloned in double-strength medium with 0.15% agar, 20% horse serum, and the same concentration of the drug used for the third selection in liquid medium [10]. A large colony was selected from the soft-agar plates and cloned twice more in soft-agar medium. A colony from the third cloning was returned to liquid medium containing a similar concentration of the drug and was expanded for storage and characterization. The sublines were routinely cultured in an atmosphere with 5% CO_2 and were cloned in a humidified incubator. The sensitivity of sublines to the agent used for selection was determined by assaying growth inhibition at 24 h after the addition of various concentrations of the drug to the suspension cultures. All drug-resistant sublines were routinely maintained in medium containing the final concentration of the drug used for selection and were removed to drug-free medium about 48 h prior to study.

Preparation of cellular proteins for electrophoresis. Cells from cultures in the logarithmic phase of growth were collected by centrifugation for 5 min at 300 *g* and either used for the preparation of cellular extracts of transferrred to Dulbecco's methionine-less medium supplemented with 5 μCi L-[^{35}S]-methionine (sp. act., 1100 Ci/mmol) and 1.5 μg unlabeled L-methionine/ml [25]. After 18 h, the labeled cells were transferred to Eagle's MEM and cultured for an addtional 4 h before collection. The cell pellets were washed three times with phosphate-buffered saline (PBS) before extraction with 1% NP-40 plus 2 m*M* PMSF in phosphate-buffered saline for 4 min while vortexing continuously at room temperature. Debris was removed by centrifugation for 10 min at 12,000 *g* and 4° C. The supernatant fluid was diluted with an equivalent volume of double-strength Laemmli buffer before being placed in a boiling water bath for 5 min [12]. Portions of the solution were removed for protein and radioactivity assay before the extracts were stored at −20° C.

Electrophoresis on polyacrylamide gels and Western transfer procedures. For one-dimensional electrophoresis, 50 μg protein or a predetermined amount of radioactivity was loaded on 12% acrylamide stacking gels above 1.5-mm-thick gels with a linear gradient of 5%–15% polyacrylamide and 0.1% sodium dodecyl sulfate [12]. The buffer for electrophoresis contained 200 m*M* TRIS-HCl, 25 m*M* glycine, and 3.5 m*M* sodium dodecyl sulfate. For two-dimensional electrophoresis, portions of the cellular extracts were placed on preequilibrated isoelectrofocusing gels, and after developing overnight the proteins were further resolved on slab gels similar to those used for one-dimensional electrophoresis [20]. The gels were stained with Coomassie brilliant blue; if the proteins were labeled with L-[^{35}S]-methionine, fluorographs were also prepared. West-

Table 1. Characteristics of drug-resistant L1210 sublines

Subline	Drug[a]	Medium[b] (μ*M*)	IC_{50} values[c]		Relative resistance[d]
			Subline (μ*M*)	L1210 (μ*M*)	
LIa5μ*M*	VM-26	5	28.6 ±0.6 (3)	22± 1 (22)	1300
L1210/0.05	VM-26	0.05	0.39±0.1 (5)	22± 1 (22)	18
L1210/0.1	VM-26	0.1	1.21±0.2 (5)	22± 1 (22)	55
L1210/VM	VM-26	5	13.4 ±1.7 (5)	22± 1 (22)	610
L1210/VP	VP-16	5	15.2 ±3.5 (5)	180±30 (3)	84
L1210/DACT	DACT	0.1	3.5 ±0.2 (4)	2.5± 0.8 (3)	1400
L1210/DOX	DOX	5	21.3 ±1.5 (4)	76±35 (3)	280
L1210/VCR	VCR	0.7	0.71±0.07 (4)	1.4± 0.1 (5)	507

[a] The drug for which resistance was selected

[b] The final concentration of drug used for selection of the subline, and the concentration added routinely to the medium in which the subline was cultured

[c] The IC_{50} value indicates the concentration of drug required to inhibit the growth of cultures by 50% at 24 h. The sublines were transferred to drug-free medium for 48 h prior to titration of growth inhibition and were in the logarithmic phase of growth when the drug used for selection was added

[d] Relative resistance indicates the ratio of the respective IC_{50} value for the resistant subline and for L1210 cells

ern transfer procedures have previously been described [19]. Aliquots of NP-40 lysates containing 40 μg protein from each cell type were examined.

Results

Resistance of L1210 sublines

L1210 sublines were independently selected for resistance to VM-26, VP-16, VCR, DOX, or DACT. The resistance of these sublines to the agent used for selection is recorded in Table 1. The LIa5μ*M* subline, previously selected as the prototype for resistance to VM-26 [23], was included in the present studies as a positive control. This subline is also cross-resistant to VP-16, VCR, DACT, DOX, and amsacrine (mAMSA). In addition, L1210 sublines were selected for resistance to 50 n*M* and 100 n*M* VM-26, and an independent selection was made for subline L1210/VM, which could be maintained, like LIa5μ*M*, in medium containing 5 μ*M* VM-26. The L1210/0.05 subline was the product of a single-step selection, and the L1210/0.1 subline was selected from L1210/0.05 in an additional step. The other sublines were selected in three discrete steps with progressively greater concentrations of the drug. Each subline was cloned three times in 0.15% agar with the agent used for selection before its establishment as a cell line.

Resistance to VM-26 was most rapidly obtained, whereas the selection for resistance to DACT was the slowest. The major delay in the selection of L1210/DACT cells occurred during cloning, and the poor growth of cells on soft agar in the presence of DACT finally led to termination of the selection at a drug concentration of 100 n*M*. The resistance of these sublines relative to that of the parental cell line ranged from 18-fold for L1210/0.05 to 1400-fold for L1210/DACT.

One-dimensional electrophoresis of cellular proteins

NP-40 extracts of L1210 cells and of the drug-resistant sublines were resolved electrophoretically on gels with a linear gradient of 5%–15% polyacrylamide (Fig. 1 A, B). Each lane in Fig. 1 was loaded with 50 μg protein and, after electrophoresis, the proteins were stained with Coomassie brilliant blue. A distinct band of protein with a mol. wt. of 22 kDa was observed in extracts of LIa5μ*M*, L1210/VM, L1210/DACT, L1210/DOX, and L1210/VCR cells. Although not as readily detectable as in extracts of the other sublines, a similar band was also observed in extracts of the 84-fold-resistant L1210/VP cells. This protein band was not observed in similar extracts from L1210 cells. Fluorographs of similar polyacrylamide gels with [^{35}S]-methionine-labeled extracts of the drug-resistant sublines also indicated a 22-kDa band that was absent in extracts of L1210 cells (Fig. 1 B). The amount of trichloroacetic acid-insoluble radioactivity in the various lanes varied from 100,000 to 500,000 cpm.

The Coomassie-stained electrophoretic pattern of proteins from L1210/0.05 and L1210/0.1 cells is presented in Fig. 2 A. Although a 22-kDa protein band is clearly evident in the positive control with the extract from LIa5μ*M* cells, a corresponding band was absent in extracts from the two other drug-resistant sublines as well as the parental cell line. The absence of this protein band was also noted in fluorographs of gels loaded with the equivalent of 1 million cpm trichloroacetic acid-insoluble material (Fig. 2 B).

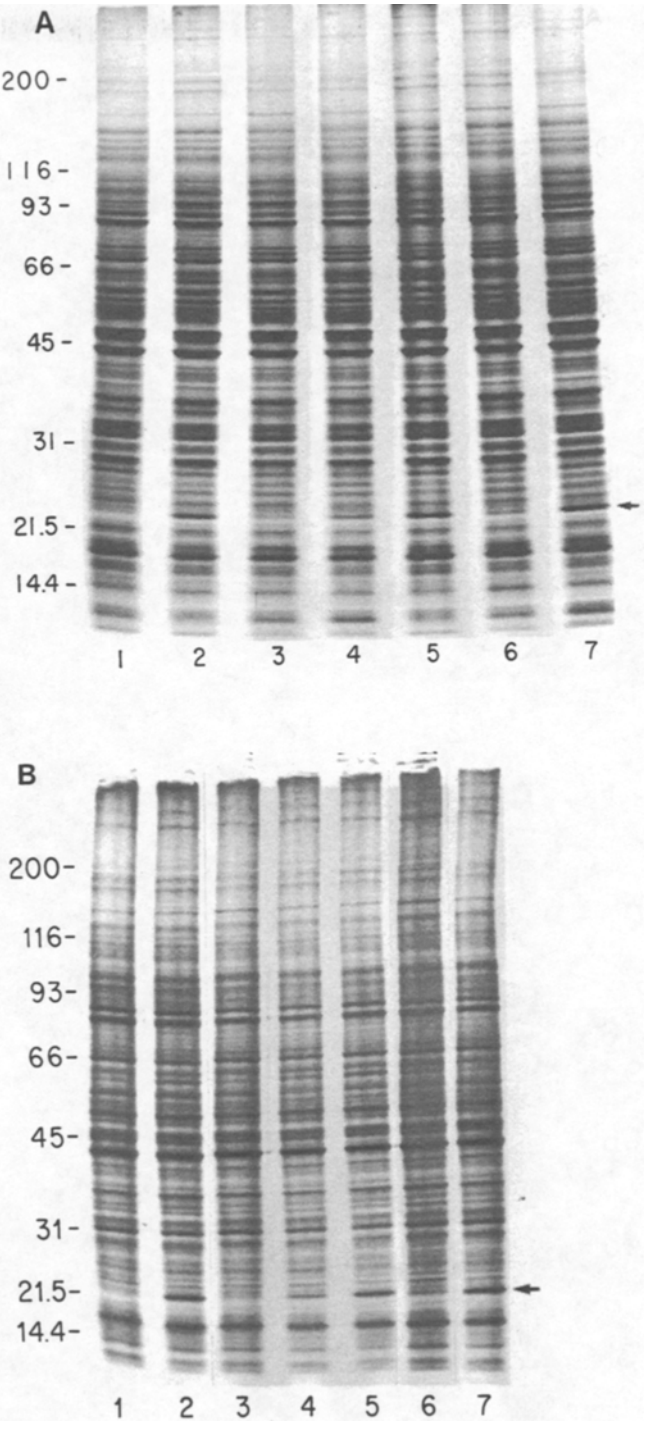

Fig. 1. Electrophoresis of NP-40 extracts on linear gradients of 5%–15% polyacrylamide. In each lane in **A**, 50 μg extract from L1210 cells or a resistant subline was loaded. After electrophoresis the gel was stained with Coomassie brilliant blue. The cell lines from which the extracts were prepared for **A B** were L1210 (*lane 1*), LIa5μ*M* (*lane 2*), L1210/VP (*lane 3*), L1210/VM (*lane 4*), L1210/DACT (*lane 5*), L1210/DOX (*lane 6*), and L1210/VCR (*lane 7*). The lanes in **B** contained 50 μg protein from cells labeled with [^{35}S]-methionine. The amount of radioactivity per lane was 149,000 cpm (*lane 1*), 136,000 cpm (*lane 2*), 140,000 cpm (*lane 3*), 102,000 cpm (*lane 4*), 219,000 cpm (*lane 5*), 503,000 cpm (*lane 6*), and 228,000 cpm (*lane 7*). The numbers to the *left* of the gels indicate the mol. wt. of protein standards. The *arrow* indicates the position of the 22-kDa protein that appeared in resistant sublines

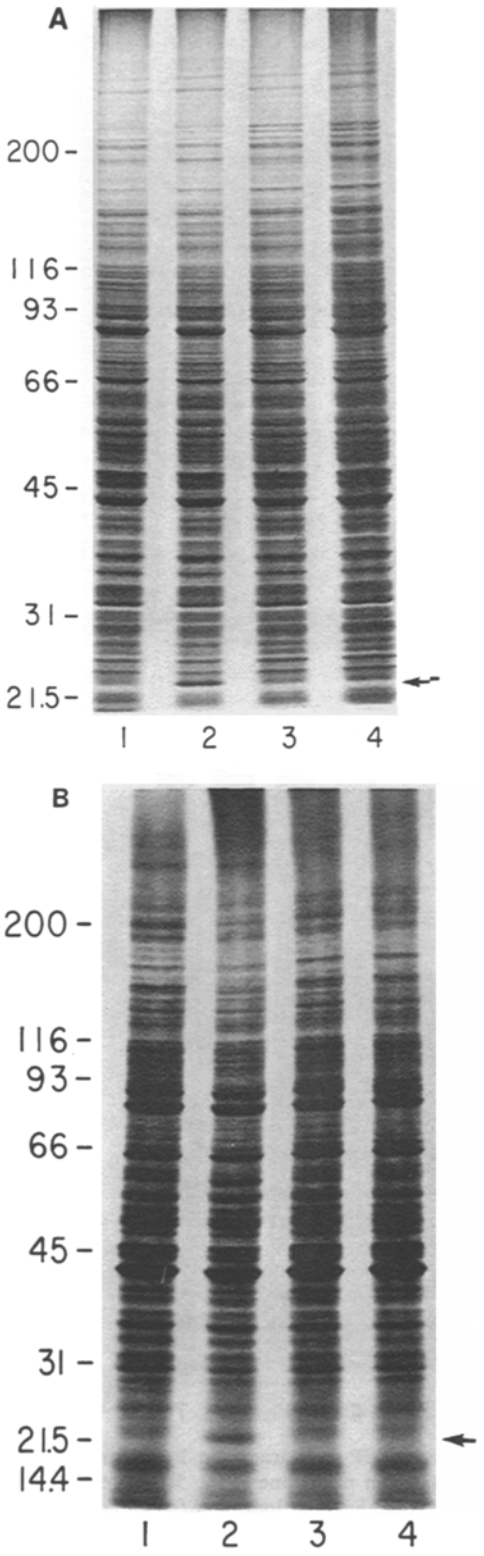

Fig. 2. The electrophoretic pattern of proteins from L1210/0.05 and L1210/0.1 cells. In **A**, 50 μg protein from NP-40 extracts of the cells was loaded per lane, and after electrophoresis the 5%–15% polyacrylamide gradient gels were stained with Coomassie blue. In **B**, proteins labeled with [^{35}S]-methionine were detected by autoradiography. Per lane, 1 million cpm from NP-40 extracts were loaded. The extracts for the gels in **A** and **B** were from L1210 (*lane 1*), LIa5μ*M* (*lane 2*), L1210/0.5 (*lane 3*), and L1210/0.1 cells (*lane 4*). The mol. wt. of the protein standards are indicated to the *left* of the gel, and the *arrow* indicates the position of the 22-kDa protein that is observed only in *lane 2* with the extract from LIa5μ*M* cells

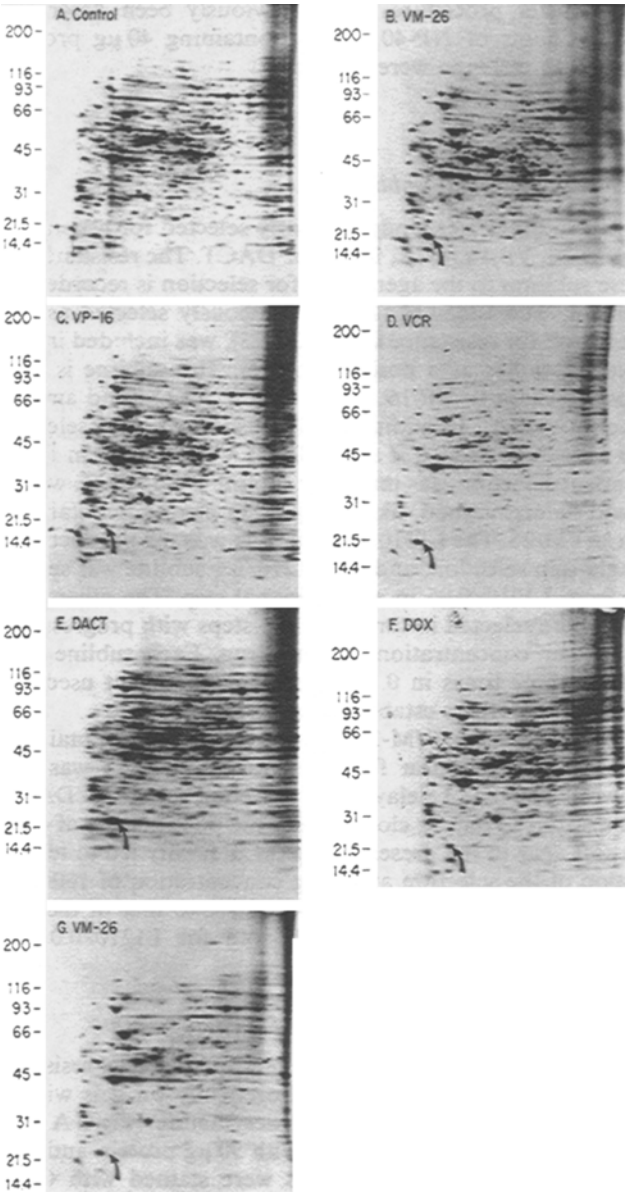

Fig. 3. Two-dimensional electrophoresis of [^{35}S]-methionine-labeled proteins from drug-resistant L1210 sublines. Autoradiographs were prepared after two-dimensional electrophoresis of 1 million cpm from NP-40 extracts of L1210 cells or sublines. The drug used for the selection of each subline is indicated in the *upper left corner* of the respective panel. The pattern for an extract of L1210 cells is presented in *panel A*. Similar extracts were also prepared from LIa5μ*M* (*panel B*), L1210/VP (*panel C*), L1210/VCR (*panel D*), L1210/DACT (*panel E*), L1210/DOX (*panel F*), and L1210/VM cells (*panel G*). The mol. wt. of protein standards are indicated to the *left* of each panel, and the more acidic proteins are found on the *left side* of the panels. The position of the 22-kDa protein is indicated by the *arrow*; its location was determined by comparison with the electrophoretic pattern of an extract from L1210 cells that was simultaneously electrophoresed

Two-dimensional electrophoretic pattern

Fluorographs of two-dimensional gels also indicated the presence of a small, anionic protein with a mol. wt. of 22 kDa in the LIa5μ*M*, L1210/VM, L1210/VP, L1210/VCR, L1210/DACT, and L1210/DOX sublines

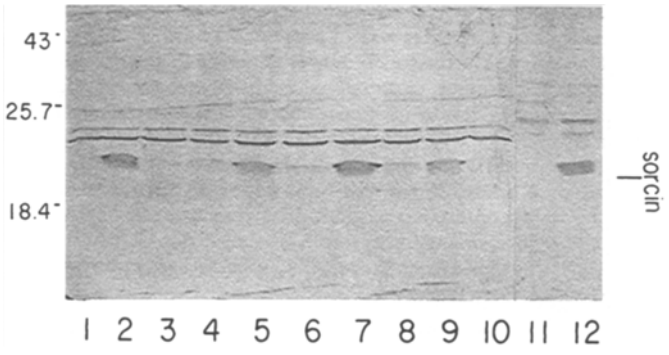

Fig. 4. Western blot with polyclonal antibody to sorcin. The mol. wt. of standards are indicated (in kDa) to the *left* of the Western blot. The cell lines from which the extracts were prepared were L1210 (*lane 1*), LIa5μ*M* (*lane 2*), L1210/0.05 (*lane 3*), L1210/0.1 (*lane 4*), L1210/VM (*lane 5*), L1210/VP (*lane 6*), L1210/DACT (*lane 7*), L1210/DOX (*lane 8*), L1210/VCR (*lane 9*), and L1210-8/800 (*lane 10*). Extracts from Chinese hamster cells were included as controls: DC-3F (*lane 11*) and DC-3F (*VCRd-5*). Subline L1210-8/800 was selected for growth in 800 n*M* VM-26 plus 8 μ*M* verapamil; it lacks the elevated levels of the 22-kDa protein

(Fig. 3). The pI values ranged between 4.9 and 6.2 for these proteins as a result of experimental variation but was generally around pI 5.5 for each of these drug-resistant sublines. For comparison, extracts from L1210 cells were developed each time a subline was tested and, as shown in Fig. 3 A, none of these gels indicated the presence of a similar, small, anionic protein in the parental cells. No corresponding protein was observed in three independent experiments with L1210/0.05 and L1210/0.1 sublines.

Identification of the 22-kDa protein as sorcin by Western transfer analysis with antisorcin antibody

Sorcin was shown by Western analysis to be overproduced in LIa5μ*M*, L1210/VM, L1210/VP, L1210/VCR, L1210/DACT, and L1210/DOX sublines compared with L1210 (Fig. 4), in accordance with observations of the overproduction of a 22-kDa protein in the resistant cells by SDS-PAGE and two-dimensional gel electrophoresis. Low-level-resistant L1210/0.05 and L1210/0.1 cells synthesized significantly less sorcin than the other resistant sublines. The amounts of sorcin in 1300-fold-resistant LIa5μ*M* and 1400-fold-resistant L1210/DACT cells were greater than in sublines with lower levels of resistance. Whether this variation in the sorcin pI (Fig. 3) among the resistant lines signifies different forms of the protein remains to be determined.

Discussion

Extracts of highly resistant L1210 sublines selected for growth in medium with VM-26, VP-16, DACT, DOX, or VCR contained a small, anionic protein that was not observed in similar extracts of the parental cells. In other studies, three revertant L1210/VM-26 sublines have been shown to lack the 22-kDa protein that characterized the cells before reversion [23]. At the time during which these revertant sublines were studied, their IC_{50} values for VM-26 remained five- to tenfold greater than that in parental cells. The present study demonstrates the antigenic identity of the 22-kDa protein with sorcin, an acidic calcium-binding protein identified in multidrug-resistant cells derived from Chinese hamster, mouse tumor, and human cell lines [17–19, 26]. The pI of the 22-kDa protein in L1210 sublines was about 5.5, close to the 5.7 value previously reported for sorcin [18]. In this group of L1210 cells, the sublines with higher levels of resistance synthesized greater amounts of sorcin than lines with lower levels of resistance. Nine L1210 sublines were independently selected for resistance to one of five different drugs, and each subline expressed a 22-kDa protein that was not observed in the parental cells. In toto, 16 drug-resistant sublines, certain of which were derivatives of the same initial selection, were cloned from the L1210 cell line, and each of these sublines expressed a 22-kDa protein. Of these sublines, the only ones that lost the 22-kDa protein were revertants grown in drug-free medium. Coincidentally, these revertants also lost the homogeneously staining regions associated with resistance [13, 23].

Low-molecular-weight proteins ranging in size from 19 to 30 kDa have been detected in multidrug-resistant human, mouse, and Chinese hamster sublines selected for resistance to VCR, DACT, DOX, or colchicine (COL) [1, 2, 5, 6, 11, 15–19, 24, 26]. The contribution of these proteins to resistance is unknown, since the overexpression of low-molecular-weight proteins does not occur in all multidrug-resistant cells [4, 17–19, 24]. The presence of a small protein in drug-resistant sublines appeared to coincide with the occurrence of gene amplification, i.e., the appearance of chromosomes with homogeneously staining regions or double-minute chromosomes [13, 17, 18]. A 21-kDa protein has been detected in murine SEWA sublines in association with double-minute chromosomes, and in one such DACT-resistant subline this protein disappeared when the cells with double-minute chromosomes were replaced by drug-resistant cells with homogeneously staining regions [16]. However, a protein analogous to the resistance-associated glycoproteins was not observed in this multidrug-resistant subline, either before or after the appearance of chromosomes with homogenously staining regions.

The overproduction of sorcin results from amplification of the gene encoding the protein in DC-3F/VCRd-5L and CH^RC5 cells [26, 27], and gene amplification is probably the basic mechanism for the overproduction of similar proteins in other lines as well. The gene for sorcin is coamplified in the above-mentioned hamster cells with the gene encoding the 150- to 180-kDa resistance-related membrane glycoprotein (P-glycoprotein) [26, 27]; coamplification may have occurred in the present L1210 sublines, since low levels of two proteins in this size range appeared in these sublines and cross-reacted with antibody to P-glycoprotein (Meyers and Roberts, unpublished observation). The probable amplification of the sorcin gene occurred with unusually high frequency among these L1210 sublines (nine independently selected lines). In a survey of multidrug-resistant Chinese hamster, mouse, and human cell lines, about 50% of the lines known to have amplified P-glycoprotein genes were found to overproduce sorcin [4]. Furthermore, it should be pointed out that since sorcin appears to be a coamplified gene (i.e., contained in the same amplicon as the P-glycoprotein gene), all of the L1210 sublines probably have amplified P-glycoprotein genes.

The role, if any, of sorcin in multidrug resistance has not been defined. Although the gene for sorcin is coampli-

fied in the same amplicon as the gene for P-glycoprotein [26], after the transfection of cells with the P-glycoprotein gene and selection for multidrug resistance, the transfected gene was amplified in the absence of amplification of the sorcin gene [7]. If sorcin does play a role in the establishment or maintenance of resistance, it may be as an auxiliary protein – perhaps one that affects the pattern of cross-resistance in cells. On the other hand, the gene for sorcin may be an integral and necessary part of the amplicon in which the P-glycoprotein gene normally resides in L1210 cells.

Characteristics of classic multidrug resistance include cross-resistance to VCR as well as to agents such as VP-16, DOX, DACT, and mAMSA that stabilize the transitional intermediate of TOPO II with DNA, the appearance of a small protein that cross-reacts with antibody to sorcin, the affinity of this protein for calcium ions, gene amplification, the preliminary evidence for the appearance of P-glycoprotein in the plasma membrane, and alterations in the flux of VM-26 across the plasma membranes of LIa5μ*M* cells. However, resistance-related changes other than those included in the multidrug resistance category may also have occurred in the LIa5μ*M* subline [14].

In support of this projection, equitoxic levels of VM-26 produced fewer lesions in the DNA of LIa5μ*M* cells than in that of L1210 cells [22]. Similarly, the progression of these resistant cells through the cell cycle was blocked less effectively than that of parental cells [23]. This pattern of multiple bases for resistance was also expressed in an ellipticine-resistant Chinese hamster cell line, in which the uptake of DOX, but not of mAMSA, was reduced and fewer lesions were induced in DNA by 2-methyl-9-hydroxyellipticinium, mAMSA, and VP-16 than in the parental cells [21]. This ellipticine-resistant subline was cross-resistant to anthracyclines, mAMSA, and VP-16. In contrast to the present VM-26-resistant sublines, a human lymphoblast subline and a Chinese hamster subline that were selected for resistance to VM-26 did not express multidrug resistance, although both sublines were cross-resistant to agents that interact with TOPO II [3, 9]. The known characteristics of the multidrug-resistant phenotype are probably not the sole basis for resistance in the LIa5μ*M* subline.

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