Identification of a novel calcium-binding protein (CP_{22}) in multidrug-resistant murine and hamster cells

Gordon Koch, Michael Smith, Peter Twentyman* and Karen Wright*

MRC Laboratory of Molecular Biology and **MRC Clinical Oncology and Radiotherapeutics Unit, Medical Research Council Centre, Hills Road, Cambridge CB2 2QH, England*

Received 15 November 1985

Analysis of cytoplasmic extracts of multidrug-resistant murine and hamster cells by SDS gel and 2D gel electrophoresis showed that they expressed an abundant 22 kDa protein which was absent from the drug-sensitive parent lines. SDS gel electrophoresis in the presence of EGTA and direct binding tests with $^{45}Ca^{2+}$ showed that the resistance-associated protein is a specific calcium-binding protein. Thus the development of multidrug resistance in both colchicine-selected hamster cells and adriamycin-selected murine cells is associated with a major change in calcium metabolism. These observations provide the first molecular basis for the hypothesis that Ca^{2+} plays a central role in the development of the multidrug resistance phenomenon.

Multidrug resistance *Cytoplasmic protein* *Calcium-binding protein* *CP_{22} protein* *Calcium antagonist* *Drug efflux*

1. INTRODUCTION

Somatic mammalian cells selected for resistance to one type of cytotoxic drug frequently develop resistance to drugs of unrelated structure and function [1,2]. The mechanisms underlying this multidrug resistance (MDR) phenomenon are unknown, but evidence exists that both decreased drug penetration [3–5] and increased drug efflux [6–19] occur in resistant cells. Decreased drug penetration appears to result from membrane changes due to increased expression of a plasma membrane glycoprotein (P-glycoprotein) [20,21]. Increased drug efflux appears to result from a Ca^{2+}-dependent process, since it is inhibited by calcium antagonists [16–19]. However, little is known about the molecular basis of this latter process. Here, it is shown for the first time that MDR cells express a novel major calcium-binding protein which could provide the molecular basis for the calcium-dependent increase in drug efflux in multidrug-resistant cells.

2. MATERIALS AND METHODS

2.1. *Cells*

CHO-AUX and CHO-C5 cells [24] were kindly provided by Dr V. Ling. EMT6 AR1 (table 1) is a

Table 1

Drug sensitivity of EMT6/Ca/VJAC cells

Drug	ID_{50} (μg/ml)	
	EMT6	EMT6-AR1
Adriamycin	0.020	0.60
Vincristine	0.013	0.62
Colchicine	0.0055	0.38

EMT6/Ca/VJAC [29] cells were grown in increasing amounts of adriamycin over 8 weeks until they became resistant to 1.0 μg/ml adriamycin (EMT6-AR1). Multidrug resistance was determined by measuring the ID_{50} (μg/ml) of drug required to inhibit cell growth by 50%. 5×10^4 cells were initiated at day 0 in the presence of the drug and allowed to grow for 3 days (parent line) or 4 days (resistant line) at which stage control samples contained 2×10^6 and 1×10^6 cells, respectively

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/86/$3.50

multidrug-resistant derivative of the murine EMT6 line [25].

2.2. *Preparation of cell extracts*

Cells were suspended in 10 mM Tris-HCl (pH 7.5) on ice for 30 min and lysed by syringing through an 18 gauge needle. Cell debris was spun out 30000 × *g* and the supernatant used as a cytoplasmic extract.

2.3. *Gel electrophoresis*

SDS-polyacrylamide gel electrophoresis was car-

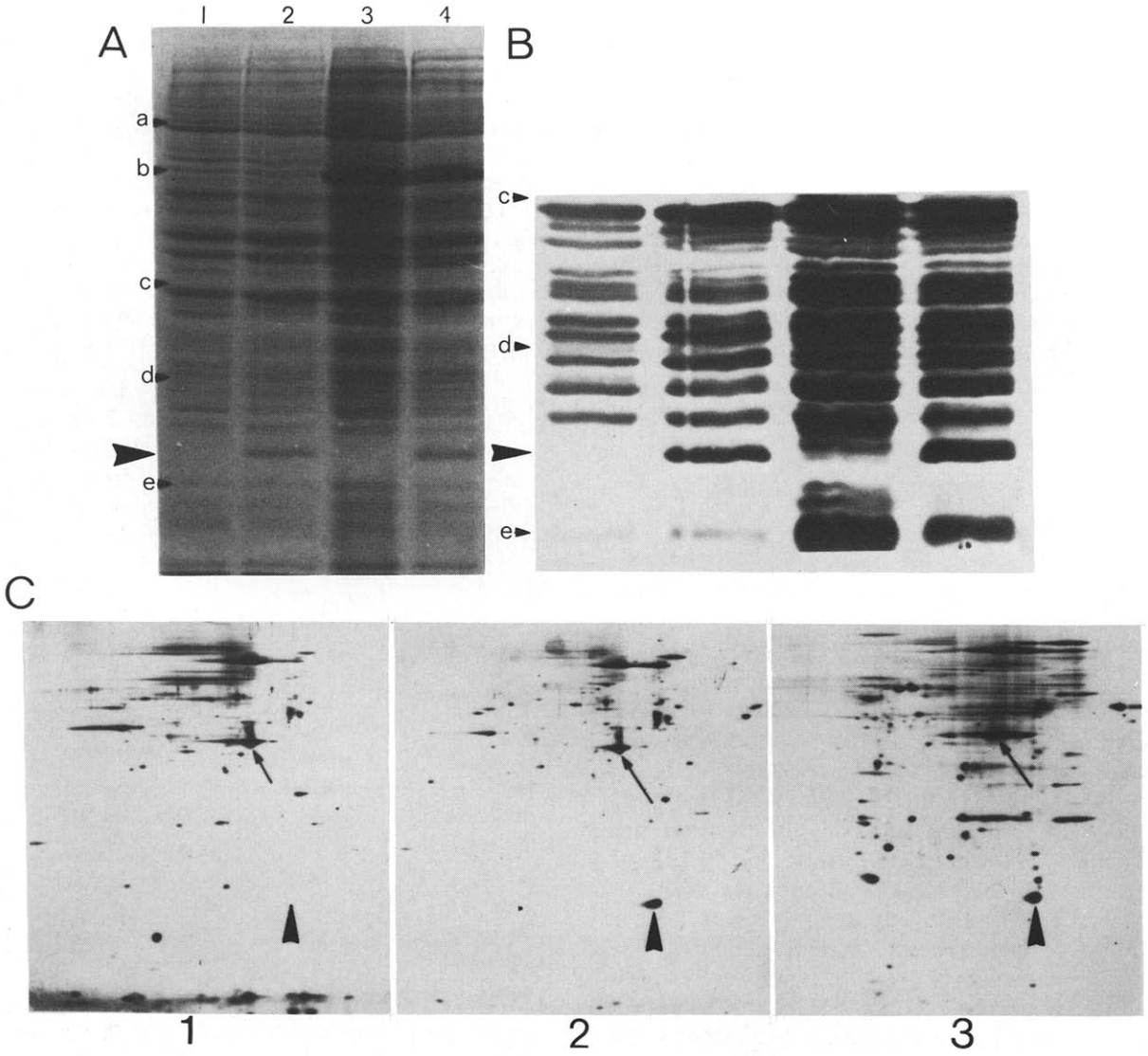
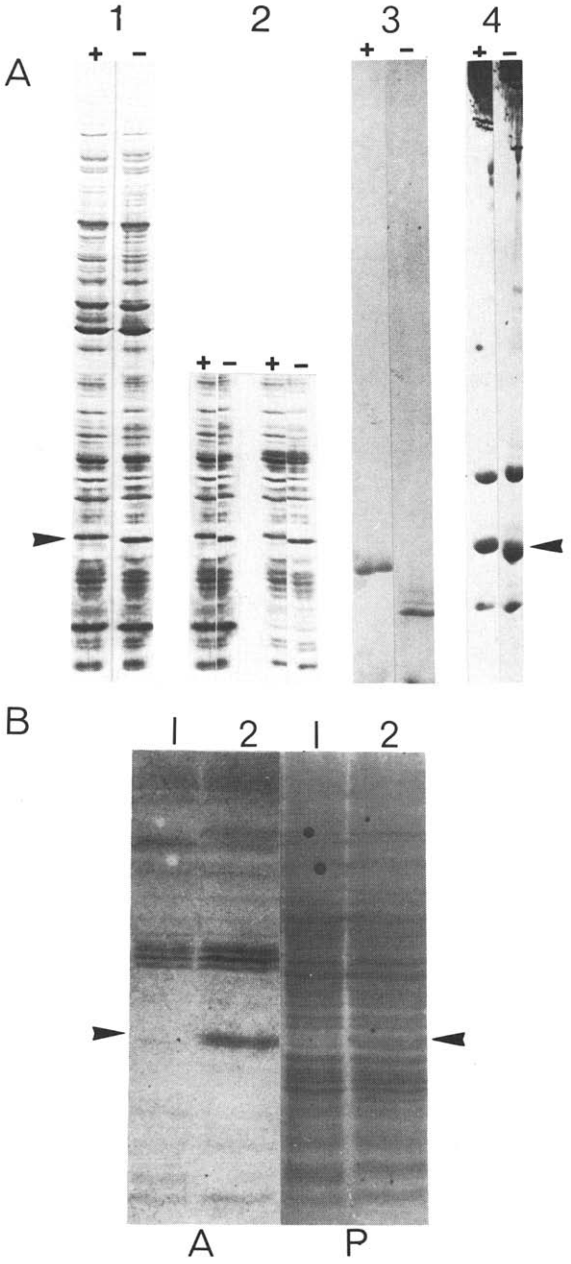


Fig.1. An abundant cytosolic protein in multidrug resistant hamster and mouse cells. (A,B) SDS-polyacrylamide gels of cell extracts from sensitive and resistant cells. (A) Gel stained with PAGE blue 83. (B) Low-molecular-mass section of the same gel as A stained with the silver stain [29]. Small arrowheads show standard markers [(a) glycogen phosphorylase, 95 kDa; (b) bovine serum albumin, 65 kDa; (c) ovalbumin, 45 kDa; (d) carbonic anhydrase, 30 kDa; (e) α-lactalbumin, 15 kDa]. The large arrowheads show the 22 kDa protein. (1) CHO-AUX, (2) CHO-C5, (3) EMT6, (4) EMT6-AR1. (C) 2D polyacrylamide gels [28] of extracts from sensitive and resistant cells. (1) CHO-AUX, (2) CHO-C5, (3) EMT6-AR1. The arrow points to actin and the large arrowhead to the position of the 22 kDa protein (p*I* 5.3).

ried out according to Laemmli [26] and Matsudaira and Burgess [27]. 2D gel electrophoresis was as described by O'Farrell [28]. Silver staining was as described by Wray et al. [29].

2.4. *Calcium binding*

This was exactly as described by Maruyama et al. [23] using $^{45}CaCl_2$ from Amersham.



3. RESULTS

SDS-polyacrylamide gel electrophoresis of extracts of the soluble proteins from the multidrug-resistant CHO-C5 and EMT6-ARI cells showed that they hyper-expressed a major 22 kDa protein, compared with the parent cell lines AUX and EMT6, respectively (fig.1). 2D gel analysis revealed that the murine and hamster proteins mapped at the same position (p*I*5.3) showing that they are the same. Thus, resistance in both these murine and hamster cells selected with either colchicine or adriamycin is associated with hyper-expression of the same 22 kDA protein.

Two lines of evidence showed that the 22 kDa protein is a calcium-binding protein. SDS gel electrophoresis in the presence of EGTA [22] (fig.2) caused a small but significant decrease in the mobility of the 22 kDa protein. The shift which occurs is less than that obtained with calmodulin which has 4 calcium-binding sites but is comparable to that of myosin light chain 2 which has only one. Confirmation of the calcium-binding capacity of the protein was obtained from a direct assay with $^{45}Ca^{2+}$ on nitrocellulose paper [23]. The 22 kDa protein was one of the major calcium-binding proteins in the extracts. No comparable activity was evident in the parent AUX line, confirming that binding was to the resistance-associated protein. When the concentration of $^{45}CaCl_2$ was varied from 1×10^{-5} to 1×10^{-7} M in the nitrocellulose binding assay, half-maximal binding occurred at 2×10^{-6} M. When the binding

Fig.2. Binding of Ca^{2+} to the 22 kDa protein in multidrug-resistant cells. (A) Analysis for calcium-binding proteins by SDS-polyacrylamide gel electrophoresis. Samples were run on mini-gradient gels (10–20% polyacrylamide) [27] in the presence (+) and absence (−) of 1 mM EGTA [22]. (1) CHO-C5 cell extract (see fig.1); (2) CHO-C5 extracts as in (1); (+) and (−) tracks were split after photography and aligned for maximum coincidence by eye, (3) bovine brain calmodulin; (4) rabbit muscle myosin (arrow shows myosin LC2, the main calcium-binding protein). The arrowheads show the position of the 22 kDa protein in CHO-C5 cells. (1) CHO-AUX, (2) CHO-C5. (A) Autoradiograph, (P) protein. Contrast in the protein stain is poor because the amido black stain was used. PAGE blue 83 is incompatible with nitrocellulose. The arrow shows the position of the 22 kDa protein.

was carried out in the presence of $MgCl_2$ from 1×10^{-6} to 1×10^{-1} M, half-maximal inhibition occurred at 10 mM $MgCl_2$. Thus the protein has a strong and specific affinity for Ca^{2+}.

4. DISCUSSION

This is the first demonstration that multidrug-resistant cells hyper-express a calcium-binding protein (CP_{22}). Although only 2 MDR cells were examined here, the expression of a similar protein (22 kDa, p*I* 5.3) has been reported in several other MDR lines [30,31]. Thus hyper-expression of the CP_{22} protein appears to be a common correlate of the MDR phenomenon, at least in rodent cells.

Two features of the CP_{22} protein are particularly significant in relation to multidrug resistance. First, the protein is easily the major change in protein composition in such cells and is therefore a major candidate product of the gene amplification events which accompany multidrug resistance [32]. It is noteworthy that this is also the first report that the CHO-C5 cell, which is the prototype line [20] for the study of P-glycoprotein expression in multidrug-resistant cells, also expresses the CP_{22} protein. Thus P-glycoprotein expression is not the only, or even the major compositional change occurring in these cells. The second significant point stems from the presence of such a major calcium-binding protein in the cytoplasm of the resistant cells. The specificity and affinity of the CP_{22} protein for calcium are comparable to that of proteins such as calmodulin and clearly implies that the protein has the ability to bind calcium in the cell. The remarkable feature of CP_{22} is the fact that its intracellular concentration is estimated at approx. 100 μM in the resistant cells. Thus by its very presence CP_{22} would substantially alter calcium metabolism in the cell since its level far exceeds the level of free calcium (approx. 1×10^{-7} M) and it could bind as much as 10% of the total cellular calcium. We therefore conclude that calcium metabolism is significantly different in the multidrug-resistant cells compared with the sensitive parents. Since CP_{22} expression is a common correlate of the development of multidrug resistance (see above), it implies that such changes in calcium metabolism are necessary, but perhaps not sufficient, for the development of the multidrug resistance phenotype.

The implication of changes in calcium metabolism in multidrug resistance is not unexpected. Studies with calcium antagonists such as the drug verapamil have shown that these agents reduce the efflux of drugs and reverse the resistance in MDR cells [10,19], indicating that Ca^{2+} is important in the drug efflux mechanism. Analysis of this phenomenon, which is of not inconsiderable clinical significance, has been difficult because the mechanism of drug efflux is unknown. The fact that CP_{22} is a calcium-binding protein provides independent evidence for the involvement of Ca^{2+} in the efflux mechanism, but more importantly, provides a biochemical handle for the examination of the drug efflux mechanism.

In summary, these studies have shown that the development of multidrug resistance in murine and hamster cells is accompanied by the expression of an abundant calcium-binding protein and thereby provides a molecular basis for the changes in calcium-dependent drug efflux which accompanies development of the resistant phenotype.

REFERENCES

[1] Dano, K. (1972) Cancer Chemother. Rep. 56, 701–708.
[2] Bech-Hansen, Till, J.E. and Ling, V.J. (1976) Cell Physiol. 88, 23–31.
[3] Dano, K. (1973) Biochim. Biophys. Acta 323, 466–483.
[4] Juliano, R.L. and Ling, V. (1976) Biochim. Biophys. Acta 455, 152–162.
[5] Baker, R.M. and Ling, V. Methods Membrane Biol. 9, 337–384.
[6] Inaba, M. and Johnson, R.K. (1977) Cancer Res. 37, 4629–4634.
[7] Inaba, M. and Johnson, R.K. (1977) Biochem. Pharmacol. 27, 2123–2130.
[8] Chitnis, M.P. and Johnson, R.K. (1978) J. Natl. Cancer Inst. 60, 1049–1054.
[9] Kessel, D., Botterill, V. and Wodinsky, I. (1968) Cancer Res. 28, 938–941.
[10] Kessel, D. and Wheeler, C. (1984) Biochem. Pharmacol. 33, 991–992.
[11] Inaba, M., Koboyashi, H., Sakurai, Y. and Johnson, R.K. (1979) Cancer Res. 39, 2200–2203.
[12] Skovsgaard, T. (1978) Cancer Res. 38, 1785–1791.
[13] Skovsgaard, T. (1980) Cancer Res. 40, 1077–1083.
[14] Dano, K. Proc. 13th Int. Cancer Congress, part C, Biology of Cancer, pp.231–246.

[15] Slater, L.M., Murray, S.L., Wetzel, M.W., Wisdon, R.M. and Duvall, E.M. (1982) J. Clin. Invest. 70, 1131–1134.
[16] Tsuruo, T., Iida, H., Tsukagoshi, S. and Sakurai, Y. (1982) Cancer Res. 42, 4730–4733.
[17] Tsuruo, T., Iida, H., Yamashiro, M. and Sakurai, Y. (1982) Biochem. Pharmacol. 31, 3138–3140.
[18] Tsuruo, T., Iida, H., Tsukagoshi, S. and Sakurai, Y. (1983) Cancer Res. 43, 2267–2272.
[19] Tsuruo, T., Iida, H., Tsukagoshi, S. and Sakurai, Y. (1983) Cancer Res. 43, 2905–2910.
[20] Riordan, J.R. and Ling, V. (1979) J. Biol. Chem. 254, 12701–12705.
[21] Kartner, N., Riordan, J.R. and Ling, V. (1983) Science 221, 1285.
[22] Burgess, W.H., Jemiolo, D.F. and Kretsinger, R.H. (1980) Biochim. Biophys. Acta 623, 257–270.
[23] Maruyama, K., Mikawa, T. and Ebashi, S. J. Biochem. (Tokyo) 95, 511–519.
[24] Ling, V. and Thompson, L.H. (1974) J. Cell. Physiol. 83, 103–116.
[25] Rockwell, S.C., Kallman, R.F. and Fajardo, L.F. (1972) J. Natl. Cancer Inst. 49, 735–749.
[26] Laemmli, U.K. (1970) Nature 227, 680–685.
[27] Matsudaira, P.T. and Burgess, D.R. (1978) Anal. Biochem. 87, 386–396.
[28] O'Farrell, P.H. (1975) J. Biol. Chem. 250, 4007–4021.
[29] Wray, W., Boulikas, T., Wray, V.P. and Hancock, R. (1981) Anal. Biochem. 118, 197–203.
[30] Meyers, M.B. and Biedler, J.L. (1981) Biochem. Biophys. Res. Commun. 99, 228–235.
[31] Polotskaya, A.V., Gudkov, A.V. and Kopnin, B.P. (1983) Bull. Exp. Biol. Med. 9, 95–96.
[32] Gudkov, A. and Kopnin, B. (1985) Bioessays 3, 68–71.