The anthracycline resistance-associated (ara) gene, a novel gene associated with multidrug resistance in a human leukaemia cell line

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Summary Multidrug resistance (MDR) in cancer cells is a major contributor to the failure of chemotherapy treatment. This paper describes a novel protein named the anthracycline resistance associated (ARA) protein. The ara gene is amplified in the MDR leukaemia line CCRF-CEM/E1000 and its mRNA is overexpressed. ARA belongs to the ATP binding cassette (ABC) family of proteins. Another ABC protein, the multidrug resistance-associated protein (MRP), has previously been reported to be overexpressed in the CEM/E1000 subline. The primary amino acid sequence of ARA indicates that it is 49.5 kDa without glycosylation, and that it has one potential glycosylation site. ARA has one ATP binding site and associated transmembrane regions. This is in contrast to MRP (190 kDa, 172 kDa deglycosylated) and most other eukaryote ABC proteins, which consist of two similar halves, each having one ATP binding site. In addition to ARA being coexpressed with MRP, comparison of amino acid sequences showed that, among known proteins, ARA is most similar to the C-terminal half of MRP.

Keywords: multidrug resistance; anthracycline resistance-associated protein

Drug resistance in cancer cells is known to have a number of different forms. A major form of drug resistance has been termed multidrug resistance (MDR), of which several types have been reported. A general characteristic of MDR cells is that they are resistant to a broad range of structurally unrelated, natural product drugs. The first MDR mechanism described in detail is attributed to overexpression of P-glycoprotein, the product of the human mdr1 gene (for review see Germann et al., 1993; Nielsen and Skovgaard, 1992). P-glycoprotein is a plasma membrane-bound glycoprotein that acts as an ATP-dependent drug efflux pump and is a member of a diverse group of membrane transport proteins called the ABC (ATP-binding cassette) protein family (for review see Higgins, 1992). ABC proteins require two nucleotide-binding regions and associated transmembrane regions to be functional. Nucleotide-binding regions of ABC proteins are characterised by conserved sequences such as the Walker A and Walker B sites (Walker et al., 1982) and the ABC signature site (Higgins et al., 1990). In eukaryotes, the two sites are usually used in two halves of a single polypeptide, while in prokaryotes ABC proteins have only one nucleotide-binding region and are therefore functional as dimers.

Another MDR mechanism involves overexpression of the multidrug resistance-associated protein (MRP) (Cole et al., 1992), which is also an ABC protein. MRP has been identified as a 190 kDa glycoprotein (Barrand et al., 1994; Krishnamachary and Center, 1993) and is reported to be located in the plasma membrane (Flens et al., 1994; Muller et al., 1994; Zaman et al., 1993) and in some cases the endoplasmic reticular membrane (Krishnamachary and Center, 1993) and endocytic vesicles (Almquist et al., 1995). The dependence of MRP-mediated MDR on ATP has been demonstrated (Leier et al., 1994; Versantvoort et al., 1994). Cells expressing MRP often show reduced drug accumulation (Davey et al., 1995; Binaschi et al., 1995; Barrand et al., 1993) and this has been reported in a cell line transfected with the MRP gene (Cole et al., 1994).

A number of recent reports have linked glutathione metabolism with MRP. Drug resistance in several MRP overexpressing lines was reversed by the addition of buthionine sulfoximine (BSO), an inhibitor of glutathione synthesis (Davey et al., 1995; Gekeler et al., 1995; Schneider et al., 1995). MRP has also been demonstrated to be a transporter of glutathione conjugates (Jedlitschky et al., 1994; Leier et al., 1994; Muller et al., 1994).

This paper describes a cDNA encoding a novel member of the ABC protein family which we have named the anthracycline resistance-associated (ARA) protein. ARA has some similarities with MRP and is coordinately expressed with MRP in the leukaemia MDR line CCRF-CEM/E1000 (E1000) (Davey et al., 1995). The possibilities for a role of ARA in the MDR phenotype are discussed.

Materials and methods

Cell lines and tissue culture

The cell lines used were the human leukaemia T-cell line CCRF-CEM (CEM) (Foley et al., 1965) and its anthracycline-selected MDR subline CCRF-CEM/E1000 (E1000) which overexpresses mrp mRNA but does not express detectable levels of P-glycoprotein or mdr1 mRNA (Davey et al., 1995). Cell cultures were maintained as previously described (Davey et al., 1995).

RNA extraction, cDNA library synthesis and Northern blotting

E1000 cells used to prepare a cDNA library were exposed to epirubicin for 18 h before total RNA was extracted (Wilkinson, 1988). Poly-A mRNA was isolated using PolyA Tract (Promega, MA, USA). A cDNA library was prepared from 2 µg of poly-A RNA using a Ribocloned cDNA Synthesis kit (Promega) in a lambda gt11 Sfi/Not vector (Promega). The amplified library was screened by plaque hybridisation using 32P-labelled pmp 10.1 (Cole et al., 1992) as probe. Conditions for screening were a 15 min prehybridisation in 7% sodium dodecyl sulphate (SDS), 0.25 M phosphate pH 7.0, 1 mM EDTA followed by overnight hybridisation in fresh solution plus denatured probe DNA. Washes were 1 x 5% SDS, 40 mM phosphate pH 7.0, 1 mM EDTA and 1 x 1% SDS, 40 mM phosphate pH 7.0, 1 mM EDTA. Hybridisation and washes were at 55°C. Positive plaques were isolated and confirmed positive over several screenings.
Analysis of cDNA clones

Restriction maps of positive cDNA clones were constructed and the identified restriction sites were used to create a series of subclones in the plasmid vector pGEM 11Zf(–) (Promega). Nucleotide sequence was determined by the dideoxy chain termination method (Sanger et al., 1977) using the Sequenase Version 2.0 DNA sequencing kit (USB, Ohio, USA) and the Stratagene Cyclist sequencing kit (Stratagene, USA). For regions of DNA with a high GC content, nucleotide sequence was determined using single strand DNA-binding protein (Promega) and the Sequenase kit.

Northern blots

Total RNA for Northern blots was extracted using guanidine thiocyanate (Chomczynski and Sacchi, 1987). An aliquot of 20 μg of total RNA was fractionated by electrophoresis through a 1.0% agarose gel containing 1.25% formaldehyde. Electrophoresis, transfer to a nylon membrane (Zetaprobe, Bio-Rad, Australia) and hybridisation at 42°C in a formamide buffer were as described by Sambrook et al. (1989).

DNA extraction and Southern blotting

Genomic DNA was extracted from cells (Sambrook et al., 1989), 10 μg of genomic DNA was digested with restriction endonucleases and the DNA fragments fractionated on a 0.5% agarose gel in TAE buffer (Sambrook et al., 1989). Fractionated DNA was transferred to a nylon membrane (Zetaprobe, BioRad) and cross-linked to the membrane by UV radiation in a Stratalinker (Stratagene). Hybridisation was as for cDNA library screening with hybridisation and washes at 65°C.

Results

The anthracycline resistance-associated (ara) cDNA

A cDNA library prepared from E1000 cells was screened at low stringency with pmrp10.1 as a probe. A number of weak positive plaques were isolated and confirmed positive by additional screening. Partial nucleotide sequence was obtained for several independent clones and these were shown to originate from the same gene. The nucleotide sequence was determined for an apparent full length cDNA which was found to be novel by nucleotide database comparison. The sequence contains an open reading frame coding for a protein which has been named the anthracycline resistance-associated (ARA) protein. The full nucleotide sequence of the cDNA (ara) has been entered into the EMBL nucleotide database with accession code X95715. A number of clones differed in their 3’ sequence, indicating the use of several polyadenylation sites. Comparison of ara sequence with the EMBL nucleotide database showed it to be most similar to the sequence of mrp (accession code L05628).

Expression of ara

The expression of the ara gene was examined by Northern blot analysis of mRNA under stringent conditions with a probe consisting of the ara 3’ coding and 3’ untranslated regions (Figure 1). A 2.2 kb band of mRNA was detected in the E1000 subline but not the parent CEM line, even after overexposure of the autoradiograph (not shown). The size of 2.2 kb is close to the expected size based on the cDNA clones characterised as being either full length or close to full length. The expression of mrp was examined by hybridisation of the same RNA samples using pmrp10.1 as probe (Figure 1). This produced a band of 6.6 kb corresponding to the expected mrp mRNA size reported previously in the E1000 subline (Davey et al., 1995). The integrity of the mRNA in the samples was determined by hybridisation with a β-actin probe. This gave a discrete band of hybridisation in RNA from CEM and E1000 cells (Figure 1), confirming the absence of detectable ara mRNA in the CEM line.

Genomic DNA analysis of the ara gene

DNA was extracted from E1000 cells and CEM cells, digested with HindIII or XbaI and analysed by Southern blot using an ara specific probe (Figure 2a). The size of the hybridisation bands was the same in CEM cells and E1000 cells. Ethidium bromide staining of the gel before transfer showed identical amounts of DNA in all lanes, and the autoradiograph results shown in Figure 2a were all obtained from the same exposure. The stronger signal in E1000 cells indicates that the ara gene has been amplified in the E1000 subline in a similar manner to that observed for the mrp gene in this subline (Figure 2b).

The anthracycline resistance-associated (ARA) protein

The ara cDNA sequence contains an open reading frame of 453 codons. The predicted molecular mass of the encoded protein based on the primary amino acid sequence is 49 561

Figure 1 Northern blot of mRNA from CEM (A) and E1000 (B) cells probed with ara (a), mrp (b) and β-actin (c) 32P-labelled probes. Molecular size markers are shown on the left.

Figure 2 Southern blot analysis of genomic DNA probed with (a) 32P-labelled ara cDNA and (b) 32P-labelled mrp cDNA. CEM (A) and E1000 (B) genomic DNA was digested with HindIII (left lane) and XbaI (right lane). All lanes in (a) are from the same autoradiograph exposure and all lanes in (b) are from the same autoradiograph exposure.
Da, and the sequence contains one potential glycosylation site (amino acids 172–175). Comparison of the ARA amino acid sequence with protein databases showed the amino acid sequence of MRP (171 562 Da unglycosylated) was the most similar. A computer alignment of the amino acid sequence of ARA and MRP is shown in Figure 3. The sequences have 51% amino acid identity and when conserved substitutions were taken into account the sequences have 71% similarity. Comparison of the ARA and MRP amino acid sequences shows some regions of higher similarity. The region centred around the Walker A site (Walker et al., 1982) has 17 identical amino acid residues and the region around the Walker B (Walker et al., 1982) and the ABC signature (S) (Higgins et al., 1990) sites have 30 out of 35 identical residues with four of the five mismatches being conserved substitutions. The identification of the highly conserved nucleotide binding site and the similarity of ARA and MRP indicate that ARA is a member of the ABC transporter family.

| I       | ARA | 1 MTEGRFCSRWLPALAIQGLPASMAVLLCGARASRLLPQLQWDDVVRSP 50 |
|--------|-----|-----------------------------------------------|
|        | MRP | 1014 VRSLVYG SAIGQGTAVFGYSAVSGGILASRCVHLVDLHSLRSMP 1061 |
| II     | ARA | 51 SFFERTPIGHLLNRFSKETTVDVPTDKLRSLWMLAYGLLEVSLLWENP 100 |
|        | MRP | 1062 SFFERTPSGLLVRHNRFSKETMVDTNVEIKLPGMLGSLPNVIGACTVILLA 1111 |
| IV     | ARA | 314 PAAPTVLSPMHLGALGLVESGASVALLQVTLQWVRNWTDLENSTVSV 183 |
|        | MRP | 1212 LFAALFAYISRHLSLALGVLVSVSYSLQVTYMLNLVRMSSEMENIVAV 1261 |
| V      | ARA | 343 VTHTLRRIISIPIQDIPFLPGSLRMNLNLQHSDAESIAWLALTVLQKAL 333 |
|        | MRP | 1362 GLHDDLKFITIPQDPFLFSGSRNLNLDPFSQYSDEEVWTSLEALRLDF 1411 |
|       | ARA | 334 VACLPGQLQOKCARGSEDLSYGRKQKLCLARALLRKTOILIDLEATAVD 383 |
|       | MRP | 1432 VSAIPDKLDHEAEGGNSLYGRKQKLYCLARALLRKTKLIDLEATAVD 1461 |
|       | ARA | 343 PGTELOMQLGMLGSGGPQCTVLLIAHRLRSVMDVLRVMDKQGVAESGSP 433 |
|       | MRP | 1462 LRTDLIQSTIRQPDFCTVLTIAHRKNMDTIVTVDKBEIQGQYP 1511 |
|       | ARA | 434 AQLAAQKGLFLYRLQAESGLV 453 |
|       | MRP | 1512 SDLQQRGLFYSMAKDLGAV 1531 |

Figure 3 Derived amino acid sequence of the anthracycline resistance-associated (ara) gene (EMBL accession code X95715) aligned with the sequence of MRP (EMBL accession code L05638). The sequences are as aligned by the program bestfit. The Walker A and B sites and the ABC signature site are underlined and labelled A, B and S respectively. Predicted transmembrane domains are underlined and are numbered with roman numerals.
A map of the characteristic functional regions of ABC proteins (ATP-binding region and associated transmembrane regions) for ARA and MRP is shown in Figure 4. ARA has only one ATP-binding site while MRP has two. ARA has five predicted transmembrane domains compared with the three MRP domains in the related regions. As indicated on the sequence in Figure 3 and shown graphically in Figure 4, ARA site I lines up with MRP site X, and MRP site IX does not have an equivalent in ARA. The next site for ARA (II) corresponds to MRP site XI, and the third ARA site (III) is closer than the next MRP site (XII) as there is an additional 67 amino acids in the sequence of MRP. ARA has two extra regions, ARA sites IV and V. Using the same prediction programme as was used for ARA, these sites are not predicted to be present in MRP.

Discussion

In this report, we have described a novel protein that we have named the anthracycline-resistance-associated (ARA) protein. Northern blot analysis showed that ara mRNA was overexpressed in the MDR E1000 subline compared with the parent CEM cell line in which ara mRNA was below the level of detection using Northern blot analysis of total RNA (Figure 1). The mRNA for mrp is also overexpressed in the E1000 subline (Davey et al., 1995). A basal level of mrp mRNA can be detected in total RNA from the CEM cells (Davey et al., 1995). The mechanism causing increased expression of each of these genes is probably related to the amplification of the genes in the E1000 subline relative to the CEM line as evidenced by Southern blot analysis (Figure 2) which shows that both are amplified to a similar extent. Amplification of the mdr gene in MDR cell lines has been previously reported (Cole et al., 1992).

Amino acid sequence comparisons indicate that ARA is a member of the ABC protein family and it is most similar to MRP (51% identical). ARA has similarity with numerous other members of the large ABC protein family. Two such proteins are the yeast proteins, YCF1 (EMBL accession P39109; Szczypta et al., 1994) and YK84 (EMBL accession P36171), each having 43% amino acid identity to ARA. The similarity to YCF1 is perhaps more significant as there are 16 out of 17 identical residues centred around the Walker A site and 27 out of 35 identical residues centred around the Walker B and ABC signature sites. Thus ARA, MRP and YCF1 sequences are highly conserved in these regions, suggesting that they may transport similar substrates. However, ARA and MRP are both associated with MDR whilst YCF1 is involved in cadmium resistance. The primary substrate for these proteins therefore appears to be diverse. However, it may be that these proteins transport substrates with an associated common factor. This may involve glutathione as this is implicated in the MDR in the E1000 subline (Davey et al., 1995) and can also be involved in heavy metal resistance (Coblenz and Wolff, 1994).

An important feature of ABC proteins is the presence of transmembrane regions. A comparison of the ARA transmembrane regions with the region of similarity in MRP shows ARA has one extra predicted transmembrane region on the carboxy terminal side of the ATP-binding region (region V, Figure 4). This region in MRP is not a predicted transmembrane region. Some ABC proteins have been reported which have the transmembrane regions on the carboxy side of the ATP-binding region (Turi and Rose, 1995), but the ATP-binding region situated in the middle of its associated transmembrane regions has not been previously reported. However, it is not known if the extra regions in ARA (IV and V) actually function as transmembrane regions as they may be precluded from associating with the membrane because of the surrounding protein secondary structure.

Whilst ARA is related to MRP by amino acid sequence comparison, a major difference between the two is the size of the mRNA and of the putative proteins. Ara mRNA was estimated to be 2.2 kb compared with mpr mRNA which is 6.6 kb (Figure 1). From the derived amino acid sequence, the ARA protein would be 49.5 kDa without glycosylation, and there is one potential glycosylation site located in a predicted hydrophilic region. ARA is most similar to the carboxy half of MRP, as shown in the aligned amino acid sequence (Figure 3). ARA, having only one nucleotide binding site and associated transmembrane regions, is different to most mammalian ABC proteins which have two functional units on a single polypeptide. Current knowledge of ABC proteins indicates that ARA would have to act as a homodimer or as a heterodimer with another ABC protein (Higgins, 1992). One possibility could be an association between ARA and MRP.

Whilst most mammalian ABC proteins reported have two ATP-binding regions and associated transmembrane regions, a number of proteins with single ATP binding site and associated transmembrane regions have been described, and these are all organelle-associated. In humans, there are two peroxisomal membrane ABC proteins, PMP70 (Kamijo et al., 1992) and ALDP (Mossner et al., 1993), and two endoplasmic reticular ABC proteins, TAP1 and TAP2 (Beck et al., 1992). TAP1 and TAP2 are involved in peptide transport into the endoplasmic reticulum (ER) (Kelly et al., 1992) for the export of peptide antigen/MHC complex to the cell surface for recognition by cytotoxic T cells. Increased TAP expression has been associated with MRP-related MDR (Izquierdo et al., 1995). Whilst more data are required to determine the function of ARA, an interesting parallel with the TAP proteins can be drawn. In the E1000 subline and other MRP-expressing cell lines, drug transport via partitioning into the endoplasmic reticulum has been hypothesised. Evidence has implicated conjugation of drug to glutathione as part of this transport process (Jedlitschky et al., 1994; Leier et al., 1994; Muller et al., 1994); hence it may be that ARA is involved in transport of a glutathione–drug conjugate across the endoplasmic reticular membrane. MRP has been located in the ER using a polyclonal antibody (Krishnamachary et al., 1994) raised against the carboxy terminal 15 amino acids of MRP, of which 9 out of the 15 are identical to ARA. Given these similarities, it is possible that ARA was also detected in the ER. This is supported by the report of a 45 kDa deglycosylated protein being observed in the ER of MDR cells, using this antibody (Krishnamachary et al., 1994).

In this report, we have identified the novel ABC protein ARA, the mRNA for which is expressed in the MDR E1000 subline and was not detected in the parent CEM cell line. The sequence similarity between ARA and MRP and their elevated expression are consistent with ARA, as well as MRP, being involved in the complex drug resistance mechanisms in the E1000 subline. Evidence pointing to the role of ARA in drug resistance includes overexpression of...
ARA in the E1000 subline, the involvement of glutathione metabolism in the MDR of the E1000 subline and the possible location of ARA in the ER membrane where it could have a role in the transport of glutathionylated drug.

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Acknowledgement

The authors are grateful to Sheridan Henness for her expert technical assistance.

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