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P-glycoprotein—A Mediator of Multidrug Resistance in Tumour Cells

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INTRODUCTION

SIMULTANEOUS RESISTANCE of tumour cells to multiple cytotoxic drugs (multidrug resistance, MDR) is a major limitation to the successful chemotherapeutic treatment of cancer. The genetic and biochemical alterations responsible for the multidrug resistance phenotype of cancer cells have been the subject of intense investigation for more than 25 years and dramatic progress has been made in the understanding of multidrug resistance-associated genes, proteins and their mechanisms of action. Because of the inherent difficulty of studying drug resistance *in vivo* (e.g. due to heterogeneity of human cancer, or difficulty in obtaining several clinical specimens from the same tumour during the course of chemotherapeutic treatment), a large number of *in vitro* models have been developed from different tissue origins and different species (reviewed in [1, 2]). In general, drug-sensitive tissue culture cells were selected in stepwise increasing, sublethal concentrations of a single cytotoxic agent (e.g. doxorubicin, vincristine or vinblastine) and acquired a phenotype of cross-resistance to a variety of structually and functionally unrelated natural product anticancer drugs (e.g. vinca alkaloids, anthracyclines, taxanes, epipodophyllotoxins) in agreement with the early observations by Kessel and colleagues [3] and Biedler and Riehm [4].

Studies on the molecular basis of MDR have revealed that multidrug-resistant cells differ from the drug-sensitive parental cells by (1) reduced accumulation of cytotoxic drugs due to decreased drug influx and/or increased drug efflux; (2) altered expression and/or activity of certain cellular proteins; and (3) physiological changes that alter the intracellular milieu (e.g. pH). Several proteins have been found to be overexpressed in multidrug resistant human cancer cells, including the multidrug resistance *MDR1* gene product P-glycoprotein, the multidrug resistance-associated protein MRP (see Loe and associates, pages 945–957), the lung resistance protein LRP which recently has been identified as the major vault protein (see Izquierdo and associates, pages 979–984), and enzymes associated with the glutathione metabolism (see O'Brien and Tew, pages 967–978). Moreover, atypical multidrug resistance has been ascribed to decreased expression or altered activity of topoisomerase II (see Nitiss and Beck, pages 958–966). Although each of these proteins has been associated with a unique profile of cellular drug resistance, the drug-resistance patterns may be partially overlapping.

Increased expression of P-glycoprotein, the product of the human *MDR1* gene, is a well-characterised mechanism used by cancer cells to evade the cytotoxic action of anticancer drugs. P-glycoprotein was discovered 20 years ago by Juliano and Ling [5] as the most ubiquitous marker in multidrug-resistant cells. P-glycoprotein ('P' refers to its proposed role in modulating cellular permeability to drugs) is a high molecular weight integral plasma membrane glycoprotein that confers multidrug resistance to mammalian cells by acting as an energy-dependent drug efflux pump (reviewed in [6–8]). The cloning of P-glycoprotein (*pgp*) or *MDR* cDNAs has revealed that P-glycoproteins are encoded by small gene families with two members in humans and three in rodents. Despite a high amino acid sequence identity of $> 70\%$ among all P-glycoproteins, *MDR* gene products are subdivided into two different classes. Class 1 consists of the drug-transporting P-glycoproteins (or multidrug transporters) which include the human *MDR1* [9], the mouse *mdr3* (or *mdr1a*) [10, 11] and *mdr1* (or *mdr1b*) [12], the hamster *pgp1* and *pgp2* [13, 14], and the rat *pgp1* and *pgp2* (or *mdr1b*) gene products [15, 16]. Class 2 (sometimes also referred to as class 3) includes the non-drug-transporting P-glycoproteins, such as the human *MDR2/3* [17, 18], the mouse *mdr2* [19, 20], the hamster *pgp3* [21], and the rat *mdr2/mdr3* [22, 23] gene products. Gene transfer experiments involving *MDR* cDNAs under the control of different eukaryotic promoters have clearly demonstrated that the expression of a class 1 P-glycoprotein [11, 24, 25] endows drug-sensitive cultured cells with multidrug resistance, while expression of human and mouse class 2 P-glycoproteins has no direct effect on cellular drug resistance [18, 20]. Class 2 *MDR* gene products are predominantly expressed in the liver bile canaliculi [20, 26–28] and the analysis of mice with an ablated *mdr2* gene has suggested that the class 2 P-glycoproteins may transport phosphatidylcholine [29], functioning as a taurocholate-dependent lipid translocase [30, 31] (reviewed in [32]).

Overexpression of class 1 P-glycoprotein causes cancer cells to become resistant to a variety of anticancer drugs (e.g. vinblastine, vincristine, doxorubicin, daunorubicin, etoposide, teniposide and paclitaxel), as well as many other cytotoxic agents (Table 1). The clinical relevance of P-glycoprotein has been of great research interest and significant evidence has been collected during the past decade to support a correlation between P-glycoprotein expression in patient tumour specimens and a poor chemotherapy treatment prognosis (see Marie and associates, pages 1034–1038; Goldstein, pages

Table 1. Compounds which interact with P-glycoprotein

Anticancer drugs	Other cytotoxic agents	MDR-reversing agents	Cyclic and linear peptides
Daunorubicin	Colchicine	Verapamil	Actinomycin D
Doxorubicin	Emetine	Nifedipine	Gramicidin D
Mitoxantrone	Ethidium bromide	Azidopine	Valinomycin
Etoposide	Puromycin	Quinidine	Yeast a-factor pheromone
Teniposide	Mithramycin	Amiodarone	*N*-acetyl-leucyl-leucyl-norleucine
Vinblastine		Reserpine	
Vincristine		Cyclosporin A	
Mitomycin C		FK506	
Paclitaxel		Rapamycin	
Actinomycin D		Progesterone	
Topotecan		Forskolin	

1039–1050). Strategies have been devised to overcome and eventually prevent multidrug resistance in the clinic. They have mainly focused on decreasing the expression and/or inhibiting the functional activity of P-glycoprotein (see Ford, pages 991–1001; Sonneveld, pages 1062–1069; Ferry, pages 1070–1081, this issue). Pharmacological reversal of P-glycoprotein-mediated multidrug resistance *in vitro* was first reported by Tsuruo and coworkers, who demonstrated that verapamil or trifluoperazin enhanced the intracellular accumulation of vincristine in a multidrug-resistant murine leukaemia cell line, thus, potentiating its antiproliferative activity [33, 34]. Since this original observation, many chemosensitisers have been described that reverse multidrug resistance. Because most of these MDR reversing agents inhibit the drug efflux function of P-glycoprotein via direct interaction with the *MDR1* gene product and some (e.g. verapamil, cyclosporine A) represent competitive substrates for P-glycoprotein-mediated drug transport, these chemosensitisers (and photoaffinity analogues thereof) are valuable tools for structure–function analyses of P-glycoprotein, as will be discussed later.

Several years ago it was recognised that the *MDR* gene family is a subfamily of a large superfamily of ATP-binding cassette (ABC) transporters [35] or traffic ATPases [36]. ABC transporters are involved in a wide range of energy-dependent transport events across intracellular or cell surface membranes, such as the extrusion of noxious compounds, the secretion of toxins, the uptake of nutrients, the transport of ions and peptides, or cell signaling. To date, more than 100 ABC transporters have been identified from different organisms, including archebacteria, bacteria, yeast, plants, insects, animals and humans (for reviews see [37–39]). A bipartite structure, consisting of a set of multiple (most often six) membrane-spanning segments and a hydrophilic nucleotide binding domain is characteristic for ABC transporters. In most prokaryotic ABC transporters the transmembrane (TM) domains and nucleotide binding folds are encoded by separate open reading frames within an operon, and non-covalently linked dimeric, trimeric or tetrameric complexes constitute functional transporters [35, 37, 38]. In eukaryotic ABC transporters a single gene usually codes for both membrane-integral part and nucleotide binding fold [37, 40].

While the TM domains differ significantly in their primary structure, the nucleotide binding folds are quite highly conserved among all ABC transporters with an overall sequence identity of approximately 30%. Two core consensus motifs, known as 'Walker A motif' and 'Walker B motif' are generally found in all ABC transporters (and many other nucleotide binding proteins) and are directly involved in ATP binding [41]. The so-called 'linker dodecapeptide' (L-S-G-G-(X)$_3$-R-hydrophobic-X-hydrophobic-A), however, immediately preceeding the Walker B motif and another short stretch known as 'centre region' are characteristic for ABC transporters only [42]. It has been hypothesised that these sequences, as part of a polypeptide loop that may undergo conformational changes upon hydrolysis of bound nucleotides, promote contact between the TM domain(s) and the nucleotide binding fold(s) [35, 36].

The widespread occurrence of ABC transporters and the high conservation of the ATP binding cassettes in particular suggest that these membrane proteins may share a common mechanism to execute their transport function, or at least that they use a similar mechanism to transduce the energy of ATP during the transport process. Thus, knowledge on the mechanism of action of P-glycoprotein as a representative of this fascinating, rapidly growing group of membrane transport proteins is of great interest. This review focuses on recent cell biological, molecular genetic and biochemical analyses that have been performed (1) to understand the topology and quaternary structure of P-glycoprotein, its biosynthesis and the role of its post-translational modifications; (2) to identify functionally important domains and amino acid residues within P-glycoprotein, to characterise its biochemical activities, and to provide models for molecular mechanism of action used by P-glycoprotein to mediate transmembrane translocation of anticancer drugs; and (3) to elucidate the physiological role of P-glycoprotein.

OVERALL STRUCTURE AND TOPOLOGY OF P-GLYCOPROTEIN

Mammalian P-glycoproteins are single chain proteins and consist of approximately 1280 amino acid residues (for reviews see [6–8]). P-glycoproteins are composed of two homologous halves each of which contains a hydrophobic, membrane-associated domain (approximately 250 amino acid residues) followed by a hydrophilic nucleotide binding fold (approximately 300 amino acid residues). A working model for the topology of human P-glycoprotein that is based entirely on hydropathy calculations is schematically illustrated in Figure 1. According to this model, both the N- and C-terminal membrane-associated domains harbour six predicted TM regions [9, 12]. The N- and C-terminus, as well as the two

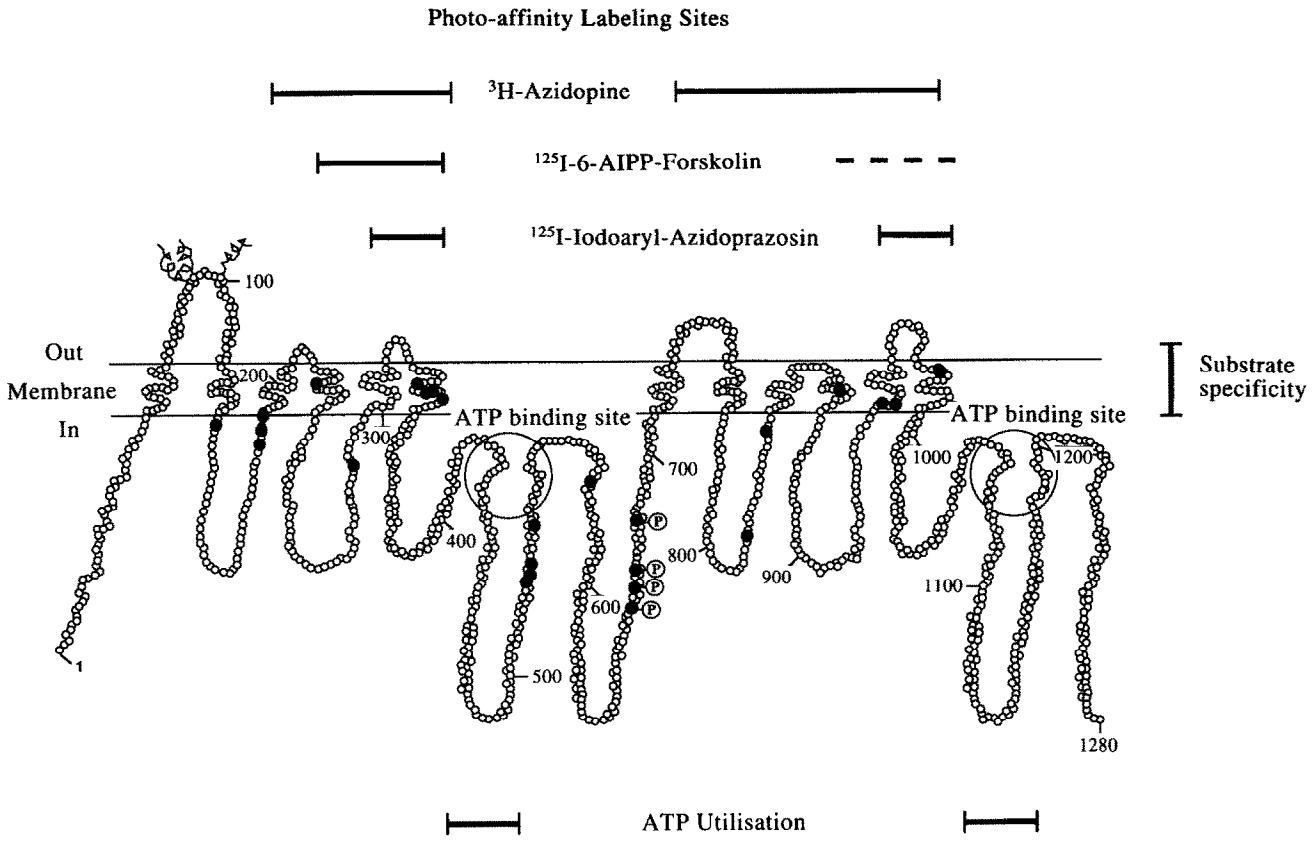

Figure 1. Schematic model of the human multidrug resistance gene product P-glycoprotein and its functional domains. A twelve-TM domain model is predicted by computer-assisted hydropathy profile analysis and amino acid sequence comparison of P-glycoprotein with bacterial transport proteins [9, 12]. Two putative ATP binding sites are circled and putative N-linked carbohydrates are represented as wiggly lines. Amino acid residues which when substituted alter the substrate specificity of the multidrug transporter are indicated in black. Phosphorylation sites are shown as a circled P. Bars point out the general region that appears to be involved in determining the substrate specificity of P-glycoprotein, the photoaffinity labelling sites, and the nucleotide binding folds. Reprinted by permission of Kluwer Academic Publishers, from Germann UA, *Cytotechnology* 1993, Vol. 12, pp. 33–62.

nucleotide binding folds are located intracellularly and the first extracellular loop is glycosylated. This twelve TM region model of P-glycoprotein is supported experimentally by cellular epitope localisation data obtained from antibodies that specifically recognise the N- or C-terminus of P-glycoprotein, its first and fourth extracellular loop, or the two ATP-binding sites [43–47]. The predicted position of the glycosylated loop has been confirmed by site-directed mutagenesis and deletion analyses [48]. However, the exact number of membrane-spanning regions and the orientation thereof has been a matter of much debate and several alternative models have been proposed for P-glycoprotein.

Based on the analyses of truncated P-glycoprotein molecules that were translated *in vitro* and translocated into microsomes, a topological P-glycoprotein variant was suggested that contains only four TM segments in each half [49, 50]. Expression studies in *Xenopus* oocytes of truncated P-glycoprotein fused to a reporter gene suggested a six TM domain configuration for the N-terminal half, and a four TM domain configuration for the C-terminal half [51–53]. Analyses of the membrane polarity of bacterially expressed P-glycoprotein-alkaline phosphatase (mdr-phoA) hybrids suggested that the N-terminal half of P-glycoprotein spans the membranes six times, but in a different manner from the model presented in Figure 1 [54], and that the putative TM domain 7 may be composed of two membrane-spanning regions, positioning the C-terminal half of P-glycoprotein asymmetrically to the N-terminal half [55]. Conversely, all studies performed with full-length, functional P-glycoprotein mutants [56] or epitope-tagged P-glycoprotein variants [57] in intact mammalian cells have corroborated the twelve TM model shown in Figure 1. It is not clear why the different heterologous expression systems or the *in vitro* translation/translocation system have failed to provide a model for the topology of P-glycoprotein that is consistent with the twelve TM domain model suggested by studies in intact mamalian cells. The reasons for the discrepancies may be linked to the fact that truncated rather than full-length P-glycoprotein constructs were studied, producing an incomplete picture since proper membrane insertion of native P-glycoprotein may depend on signals from both halves of the molecule. It has been hypothesised by Zhang and colleagues [50] that alternate topological forms of P-glycoprotein might reflect different functional phenotypes. In this respect it is interesting to note that recombinant mouse *mdr1b* P-glycoprotein mutants with substitutions in the ATP binding sites, which are unable to confer drug resistance in mammalian cells [58], are capable of transporting organic cations similar to wild-type *mdr1b* P-glycoprotein when expressed in bacteria [59].

Generally, internal hydrophobic signal sequences within polytopic membrane proteins act in a polar manner from the N-terminus to direct the insertion of the polypeptide chain into the endoplasmic reticulum [60, 61], and signal recognition particle (SRP) docking protein-dependent signal sequences have indeed been detected in both halves of P-glycoprotein [49, 51–53, 62]. Besides intragenic signal sequences, cytoplasmic factors have also been suggested to play a role in regulating the expression of topological P-glycoprotein structures, but these factors remain to be identified in various cell types and/or expression systems [62]. Thus far, the molecular chaperone calnexin has been implicated in the maturation of recombinant human P-glycoprotein transmembrane domains [63].

Twelve TM domains and two nucleotide binding folds constitute a minimal functional unit for many mammalian ABC transporters and, generally, it has been assumed that the multidrug transporter acts as a monomer. Recently, however, the existence of oligomeric forms of P-glycoprotein has been demonstrated in multidrug resistant cells. This raises the possibility that the functional activity of P-glycoprotein may be modulated via its incorporation into homo-oligomeric complexes. Indirect evidence for P-glycoprotein dimers in the plasma membranes has been obtained from radiation inactivation and freeze fracture studies [64–67]. Data from chemical crosslinking studies are also consistent with the occurrence of P-glycoprotein dimers in multidrug resistant cells [68]. Moreover, the biochemical isolation of radiolabelled P-glycoprotein from postnuclear lysates by velocity sedimentation has indicated that a large percentage of P-glycoprotein forms oligomeric complexes in an early biosynthetic compartment, the endoplasmic reticulum [69]. Oligomer formation did not require asparagine-linked glycosylation and P-glycoprotein dimers and oligomers were able to bind drug- or ATP-analogues [69]. The hypothesis that the formation and dissociation of P-glycoprotein oligomers may modulate its functional activity is attractive, but clearly requires support by additional experiments. For example, reconstitution of different forms of P-glycoprotein in protein lipid bilayers may allow us to determine if there are functional differences between monomers, dimers and oligomeric complexes.

Further studies will also need to address the dynamics of the P-glycoprotein oligomerisation process which are not understood at present. Can monomers that are integrated into the plasma membranes associate with each other to form oligomers? If so, what determines the pool size of monomers in cancer cells and what is the stimulus for oligomerisation? Are P-glycoprotein oligomers stable structures, or can they dissociate into monomers? Is the half-life of P-glycoprotein affected by its quaternary structure? Are either monomers or oligomers preferred subjects for degradation?

BIOSYNTHESIS OF P-GLYCOPROTEIN

Consistent with its suggested function as an ATP-driven efflux pump for a great variety of amphiphilic cytotoxic agents, immunocytochemical localisation studies have demonstrated that human P-glycoprotein is predominantly localised in the plasma membrane at the cell surface of multidrug-resistant cells [70]. Low amounts of P-glycoprotein have also been found in association with organelles that are involved in the processing of glycosylated, integral membrane proteins with multiple membrane-spanning regions, for example the endoplasmic reticulum or the Golgi apparatus. Although cell surface localisation of recombinant P-glycoprotein has also been observed in heterologous expression systems, for example in human *MDR1* baculovirus-infected insect cells [71] or in yeast overexpressing human P-glycoprotein [72], an unusually large fraction of recombinant P-glycoprotein appears to be present in intracellular membranes, e.g. Golgi-like vesicles or the nuclear envelope. It has been suggested that this may reflect an inefficiency of the cellular translocation machinery in processing the overexpressed foreign gene product (reviewed in [73]). At present, only a few factors that are directly involved in the biogenesis of P-glycoprotein and its translocation to the cell surface are known. A recent study on the biosynthesis of temperature-sensitive mutants carrying substitutions of amino acids located within or immediately N-terminal to the TM segment 7 has revealed that core-glycosylated P-glycoprotein in the endoplasmic reticulum is associated with the molecular chaperone calnexin and that the duration of this association is critical for the fate of the polypeptide chain [74]. While wild-type P-glycoprotein escapes the association with calnexin efficiently and is properly targeted to the plasma membranes via the Golgi apparatus, misfolded mutants remain in association with calnexin for prolonged periods in the endoplasmic reticulum [74]. A study of the association of molecular chaperones with the TM domains and nucleotide binding folds of P-glycoprotein expressed as separate polypeptides suggested that calnexin is involved in the folding of both the N- and C-terminal TM domains, whereas Hsc70 may participate in the folding of the two ATP binding domains [63]. The exact sites within P-glycoprotein that interact with calnexin or Hsc70 have not been characterised yet, and the role of these and possibly other molecular chaperones during the biogenesis of P-glycoprotein is presently unclear.

GLYCOSYLATION OF P-GLYCOPROTEIN

Soon after P-glycoprotein was discovered [5], it was reported to be a phosphoglycoprotein [75] and the role of these post-translational modifications for the drug transport function of P-glycoprotein has been addressed by numerous cell biological, biochemical and molecular genetic analyses.

P-glycoprotein is synthesised as a non-glycosylated precursor with an apparent molecular size of 120 000–140 000, which is processed to the mature form with a half-life time $t_{1/2}$ = 1–2 h in human cells or $t_{1/2}$ = 20–30 min in mouse cells [76, 77]. Mature P-glycoprotein has a molecular size that may range from 130 000–180 000 depending on the type of cell and species in which it is expressed [76, 77]. Tunicamycin inhibits processing of the P-glycoprotein precursor to the mature form, and endoglycosidase F converts mature P-glycoprotein to a lower molecular weight form that is similar to the precursor, suggesting that glycosylation of P-glycoprotein is *N*-linked [76, 78]. The primary structures of mammalian P-glycoproteins predict two, three or four *N*-linked glycosylation sites that are located in the N-terminal half within the first extracellular loop (Figure 1) [9, 11, 12, 15, 79, 80]. Asn-91, Asn-94 and Asn-99 have been confirmed as glycosylation sites within human P-glycoprotein [48]. Additional glycosylation sites have been postulated based on coupled transcription–translation–translocation analyses of P-glycoprotein fragments and supplementary studies in *Xenopus* oocytes [49–51], but no such sites have been detected by *N*-glycanase (a mixture of peptide-*N*-glycosidase F and endoglycosidase F) treatment of wild-type human P-glycoprotein or several mutants that lack

glycosylation sites in the first extracellular loop [48]. Similarly, the analysis of individual P-glycoprotein domains expressed as separate polypeptides suggested that only the N-terminal transmembrane domain was core-glycosylated [63], consistent with the study of proteolytic fragments obtained from ^{3}H-azidopine-labelled human P-glycoprotein which did not reveal a glycosylated segment in the C-terminal half [78, 81]. It has been demonstrated that ^{14}C-labelled N-acetylglucosamine and galactose are efficiently incorporated into human P-glycoprotein, while poor labelling is achieved with ^{14}C-labelled fucose and mannose [76], but the precise composition of the carbohydrate moiety of P-glycoprotein remains unknown.

Many studies have been reported which suggest that glycosylation of P-glycoprotein is not essential for its basal drug transport function. A variety of recombinant P-glycoproteins produced in heterologous expression systems (e.g. baculovirus-infected insect cells and *Saccharomyces cerevisiae*) were found to be underglycosylated or non-glycosylated, but maintained characteristic functional activities, such as ATP- and drug-binding activities and drug-stimulated ATPase activity [71, 72, 82–84]. Multidrug resistant sublines could be established from partially glycosylation-defective, lectin-resistant hamster cell mutants [85]. Similarly, a human colon carcinoma cell line known as Moser cell line, synthesising an aberrant P-glycoprotein with an altered carbohydrate moiety, was found to display a competent multidrug resistance phenotype [86]. Moreover, tunicamycin treatment of several multidrug resistant cell lines did not decrease their drug resistance [87–89], suggesting that glycosylation is not essential for sustaining the multidrug resistance phenotype. However, tunicamycin treatment of a human colon carcinoma cell clone resulted in reduced levels of cell surface-associated multidrug transporter, suggesting that glycosylation is required for efficient translocation of P-glycoprotein to the plasma membranes [90]. This is in agreement with the most conclusive study of Schinkel and colleagues [48] involving a series of human P-glycoprotein mutants in which one, two or all three *N*-linked glycosylation sites in the first extracytoplasmic loop were abrogated. Upon transfection, these glycosylation-defective P-glycoprotein mutants endowed drug-sensitive cells with the same pattern of cross-resistance as the wild-type P-glycoprotein, independent of their state of glycosylation [48]. However, a P-glycoprotein mutant lacking all three N-glycosylation sites, yielded drug-resistant transfectants with much lower efficiency than the wild-type P-glycoprotein, and based on this observation it was proposed that the carbohydrate moiety contributes to the correct folding and/or proper routing of P-glycoprotein, and/or its stabilisation en route to or within the plasma membranes [48]. Studies on the association of glycosylation-defective mutants in comparison with wild-type P-glycoprotein with molecular chaperones (e.g. calnexin [63, 74],) may help in elucidating the exact role of the carbohydrate moiety of P-glycoprotein.

PHOSPHORYLATION OF P-GLYCOPROTEIN

Phosphorylation of P-glycoprotein has been observed in many multidrug-resistant human and rodent cell lines (reviewed in [91, 92]) and similar to native *MDR* gene products, recombinant P-glycoproteins are phosphorylated [71, 93], suggesting that phosphorylation of P-glycoprotein may be universal and catalysed by a ubiquitous protein kinase(s). The role of phosphorylation of P-glycoprotein and the identification of P-glycoprotein-specific protein kinases has been of great interest to many investigators, in particular because phosphorylation/dephosphorylation mechanisms have been suggested to regulate the drug efflux activity of P-glycoprotein [75, 94–96], providing a potential target for the development of MDR modulators.

One obvious approach to evaluating the role of phosphorylation of P-glycoprotein has been to correlate the levels and/or activities of protein kinases in multidrug-resistant cells with the state of phosphorylation of P-glycoprotein, with the relative drug resistance of the cells, and with intracellular drug accumulation. Such studies have demonstrated that brief exposure to phorbol ester protein kinase activators enhanced phosphorylation of P-glycoprotein, reduced intracellular drug accumulation, and increased drug resistance in a number of multidrug-resistant cells [97–102]. Conversely, treatment of multidrug-resistant cells with the protein kinase inhibitors staurosporine and calphostin C reduced phosphorylation of P-glycoprotein and enhanced intracellular drug accumulation [98, 101–103], supporting the hypothesis that the state of phosphorylation of P-glycoprotein may regulate its drug export function and modulate multidrug resistance.

However, the activators and inhibitors of protein kinases used in these studies are not very specific and may have multiple effects in cells, which makes interpretation of the data difficult. For example the phorbol ester tumour promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA), as well as the protein kinase C (PKC) activator diacyl glycerol were found to increase *MDR1* gene expression at the transcriptional and translational levels, and staurosporine inhibited these effects [104]. It has also been reported that staurosporine has dual/opposite effects on levels of *MDR1* mRNA and P-glycoprotein depending on its concentration and the cell type investigated [104, 105]. Moreover, staurosporine and analogues thereof can reverse multidrug-resistance *in vitro*, most likely via direct interaction with P-glycoprotein since reversal of multidrug resistance was independent of their protein kinase inhibitory activities [106–110]. Similarly, calphostin C and certain isoquinolinesulphonamide (H7) derivatives may increase drug accumulation in multidrug-resistant cells by directly affecting the drug efflux activity of P-glycoprotein [108, 110, 111]. Hence, there is considerable evidence that many protein kinase modulators may alter drug accumulation within multidrug-resistant cells independent of, or in addition to their effects on the state of phosphorylation of P-glycoprotein either via direct interaction with P-glycoprotein by blocking its drug transport activity, or by altering levels of expression of the *MDR1* gene product.

Recently, agents have been described that inhibit phosphorylation of the multidrug transporter without competing directly for drug binding by P-glycoprotein and without affecting its expression levels. One such compound is safingol, a lisosphingolipid PKC inhibitor, which enhanced the cytotoxicity of vinca alkaloids and anthracyclines by increasing drug accumulation in MCF7-DOX(R) cells and concomitantly inhibited basal and phorbol-12,13-dibutyrate stimulated phosphorylation of P-glycoprotein [112]. Despite its effectiveness as an inhibitor of PKC in both drug-sensitive and multidrug-resistant cells, safingol only partially reversed multidrug resistance. The *N*-myristoylated pseudosubstrate peptide inhibitor P1 of PKCα is another agent which reduced P-glycoprotein phosphorylation and partially reversed multidrug resistance of MCF-7 breast cancer cells by enhancing the accumulation of cytotoxics [113]. P1 was not a substrate for

transport by P-glycoprotein and did not inhibit ^{3}H-azidopine photoaffinity labelling of P-glycoprotein, but actually increased ^{3}H-azidopine labelling, suggesting that the mechanism of reversal of multidrug resistance by P1 may still involve interactions with P-glycoprotein.

The identification of protein kinase(s) responsible for phosphorylation of P-glycoprotein is a necessity to fully understand the role of this post-translational modification. Since the 140 kD P-glycoprotein precursor is not phosphorylated in the resting state [86] and the mature protein is integrated in the plasma membranes, protein kinases that act at the cell surface are the most likely candidates. At least three different novel kinases have been implicated to play an important role in P-glycoprotein expressing multidrug-resistant cells ([114–116]; S.V. Ambudkar, personal communication). These kinases are membrane-associated and two of them have been shown to phosphorylate P-glycoprotein *in vitro*. Other kinases that phosphorylate P-glycoprotein *in vitro* include cAMP-dependent protein kinase (PKA) [117] and PKC [97, 98]. PKC is often increased in multidrug-resistant cells in comparison with drug-sensitive parental cells (reviewed in [91, 92]). *In vitro* phosphorylation of P-glycoprotein by PKC has been demonstrated using plasma membranes isolated from multidrug-resistant human KB-V1 cells, partially purified P-glycoprotein, or synthetic and bacterially expressed P-glycoprotein peptides [116, 118–120]. Transfection experiments have demonstrated that overexpression of PKC, the isoform PKCα in particular, is associated with enhanced phosphorylation of P-glycoprotein and increased cellular drug resistance [99, 121], and the ability of PKCα to phosphorylate P-glycoprotein and to modulate its functional activity has been well documented [121, 122]. However, our present insights concerning the expression and the role of these different protein kinases in the P-glycoprotein-mediated multidrug-resistance phenotype are rather limited. Multiple kinases may be involved in regulating P-glycoprotein expression and/or activity depending on the species, origin, cell type, and/or state of differentiation. Ultimately, the individual contributions of the different protein kinases associated with the multidrug-resistance phenotype will have to be assessed in a designated cell line and correlated with expression levels of P-glycoprotein and its state of phosphorylation. Also the proteins that dephosphorylate P-glycoprotein will have to be identified to establish the enzymology and the kinetics of the phosphorylation/dephosphorylation cycle of P-glycoprotein. Membrane-associated protein phosphatases (e.g. protein phosphatases 1 and 2A, [98]) are likely to be involved in dephosphorylation of P-glycoprotein.

Phosphoamino acid analyses of human P-glycoprotein have revealed the exclusive presence of phosphoserine [94, 95]. When human P-glycoprotein expressed in multidrug-resistant cells was metabolically labelled with ^{32}P-orthophosphate and subjected to two-dimensional tryptic phosphopeptide mapping, at least three major phosphopeptides were observed [98]. The same two-dimensional map of tryptic phosphopeptides was obtained after *in vitro* phosphorylation of human P-glycoprotein by PKC [118]. These three tryptic phosphopeptides were isolated and subjected to amino acid sequence analysis which indicated serine 661, serine 671, and one or more of serine 667, serine 675 or serine 683 as PKC phosphorylation sites in human P-glycoprotein [118]. A synthetic peptide encompassing amino acid residues 656–689 allowed the identification of serine 667 as a third PKC phosphorylation site in human P-glycoprotein [119]. A peptide with the major phosphorylation sites mutated into alanines was found to be *in vitro* phosphorylated by PKC at serine 683 [116]. Serine 667, serine 671 and serine 683 were also shown to be phosporylated by PKA *in vitro* using the synthetic P-glycoprotein peptide ranging from amino acids 656–689 as a substrate [119]. Experiments with a novel membrane-associated protein kinase isolated from multidrug-resistant KB-V1 cells ('V-1 kinase') and a series of glutathione-S-transferase (GST) fusion proteins containing amino acids residues 644–689 of the human *MDR1* gene product corroborated serine 661 and serine 667 as major phosphorylation sites in human P-glycoprotein [116]. Hence, despite the presence of numerous (>40) consensus sites for PKC and/or PKA phosphorylation distributed throughout the primary structure of human P-glycoprotein, a cluster of maximally four serine residues is phosphorylated by multiple kinases. Interestingly, this phosphorylation site cluster is confined to a central cytosolic segment that connects the two homologous halves of P-glycoprotein and is usually referred to as the linker region. In the mouse *mdr1b* gene product, there are two serines in the linker region at positions 669 and 681 (analogous positions of serine 671 and 683 in human *MDR1* gene product) which are phosphorylated by PKC and PKA, respectively [123]. Other class 1 mammalian P-glycoproteins (the mouse *mdr1a* and the hamster *pgp1* and *pgp2* P-glycoproteins) also harbour PKC and PKA consensus phosphorylation sites in the linker region, but the actual sites of phosphorylation have not yet been determined.

In analogy with the role of the highly phosphorylated R-domain of the cystic fibrosis TM conductance regulator (CFTR), which contributes to the regulation of its cAMP-dependent chloride channel activity [124–126], it has been hypothesised that the linker region of P-glycoprotein may act as a 'mini regulatory domain' to control its drug efflux activity [118]. This hypothesis was tested by a recent mutational analysis, in which the serine residues at positions 661, 667, 671, 675 and 683 in human P-glycoprotein were substituted either with non-phosphorylatable alanine residues (5A-mutant), or with aspartic acid residues to mimic permanently phosphorylated serine residues (5D-mutant) [127]. Transfection studies revealed that both the 5A- and 5D-mutant were not phosphorylated *in vivo*, but conferred multidrug resistance with similar efficiency as wild-type P-glycoprotein [127]. Reconfirming the presence of the major phosphorylation sites in the linker region of human *MDR1* gene product, this study suggests that the phosphorylation-defective P-glycoprotein variants are able to execute basal drug efflux activity and that phosphorylation/dephosphorylation mechanisms do not play a crucial role in the establishment of P-glycoprotein-mediated multidrug resistance. Similar conclusions were drawn from a study using bryostatin 1, which decreased P-glycoprotein phosphorylation, but did not affect its drug efflux activity in multidrug-resistant human breast cancer cells [128]. Although it cannot be ruled out that phosphorylation of P-glycoprotein and/or protein kinase interactions have direct or perhaps indirect modulatory effects on the drug resistance pattern of P-glycoprotein-expressing cells [100], on the kinetics of the drug transport [120], or on the stability and/or instability of P-glycoprotein [129], it is conceivable that phosphorylation of P-glycoprotein may be less significant for export of drugs, but crucial for the transport of a putative physiological substrate(s) that still remain(s) to be identified. A recent study suggests

that phosphorylation of human P-glycoprotein may modulate the regulation of an endogenous chloride channel whose identity is still obscure at present [130, 131].

DRUG AND MDR MODULATOR INTERACTIONS OF P-GLYCOPROTEIN

The most unusual and puzzling feature of P-glycoprotein is its promiscuity as a transporter. As already pointed out in the introduction, P-glycoprotein interacts with structurally and functionally unrelated molecules, and most of these compounds are substrates for transport by P-glycoprotein. All the MDR reversing agents listed in Table 1 directly interact with P-glycoprotein and block its drug efflux activity. Some of them (e.g. verapamil, cyclosporine A, azidopine, diltiazem and FK-506) are substrates for transport by P-glycoprotein [132–135]. Other MDR modulators (e.g. progesterone and nitrendipine) are not transported by P-glycoprotein and may reverse multidrug resistance by blocking the initial binding of the anticancer drug(s) to P-glycoprotein [136, 137]. Hence, besides drug substrates, MDR modulators (and their photoaffinity analogues) are valuable tools for structure–function analyses of P-glycoprotein.

The identification of protein domains and/or amino acid residues implicated in recognition, binding, transport through the membrane lipid bilayer, and release of drugs or MDR modulators by P-glycoprotein is a necessary prerequisite to understanding its structure, function and mechanisms of action. Major questions include the number, location and nature of the drug interaction sites. Are both halves of P-glycoprotein involved in drug binding? If so, can the halves of P-glycoprotein operate independently of each other? Do different drugs and/or modulators bind to the same site within P-glycoprotein or do they bind to different sites? If drugs bind to different sites, are there allosteric interactions between these sites? Are there central amino acid regions and/or residues within the P-glycoprotein polypeptide chain that are essential for the interaction with all substrates for transport? Two major approaches towards this goal have involved classical biochemical analyses of P-glycoprotein peptides labelled with analogues of MDR drugs that can be photoactivated, and modulators, and genetic analyses of P-glycoprotein mutants that demonstrate altered substrate specificity. These studies have been complemented by structure–activity relationship (SAR) studies using drug and/or modulator analogues to determine the structural features that are recognised by P-glycoprotein, and to investigate if there is a common 'MDR pharmacophore' (see Ford, pages 991–1001).

The finding that radioactively labelled cytotoxic drugs (e.g. vinblastine) or MDR-reversing agents (e.g. verapamil) specifically interact with a plasma membrane protein expressed in multidrug-resistant cells [138] triggered the synthesis of a photoactive analogue of vinblastine (I^{125}-NASV) which was used to demonstrate specific cross-linking to P-glycoprotein upon UV-exposure [139, 140]. Many different radioactive photoaffinity analogues of drugs and MDR-reversing agents have since been used to prove the drug-binding capacity of P-glycoprotein, including derivatives of vinblastine [139], colchicine [141, 142], verapamil [143], daunomycin [144], azidopine [44, 78, 81, 145–147], forskolin [148], prazosin [149], dexniguldipine [150] and others (reviewed in [151, 152]). Moreover, competition assays involving a photoaffinity analogue (e.g. the commercially available compounds ^{3}H-azidopine or ^{125}I-iodoarylazido prazosin) and a non-radioactive drug or MDR-reversing agent added in excess are a common experimental approach used to identify P-glycoprotein-interacting compounds. Data from such analyses have suggested that different drugs (for example vinblastine and colchicine) bind to separate, possibly overlapping or allosterically coupled sites, or alternatively, that P-glycoprotein harbours a common drug-acceptor site that displays variable affinity for different agents (reviewed in [152]). It needs to be pointed out that the apparent affinity of P-glycoprotein to chemosensitisers measured in such competition assays does not necessarily correlate with their potency as MDR-reversing agents measured *in vitro*.

Attempts to map putative drug-binding sites within the primary structure of P-glycoprotein have involved the immunological identification of radioactive P-glycoprotein peptides obtained by enzymatic or chemical degradation of P-glycoprotein labelled with ^{3}H-azidopine, 6-O-{{2-{3-(4-azido-3-^{125}I-iodophenyl)propionamido}ethyl}carbamyl}forskolin (^{125}I-6-AIPP-forskolin) or ^{125}I-iodoaryl azidoprazosin [44, 78, 147, 149, 153, 154]. These studies have suggested that both halves of P-glycoprotein contribute to drug binding. For ^{125}I-iodoaryl azidoprazosin two major labelling sites have been identified within the mouse *mdr1b* P-glycoprotein: one in a region near or within TM6 in the N-terminal half, and the other at an analogous position (near or within TM12) in the C-terminal half of P-glycoprotein [153] (Figure 1). Interestingly, highly related, if not the identical regions within the human P-glycoprotein are labelled by ^{3}H-azidopine [78] and ^{125}I-6-AIPP-forskolin [154] (Figure 1). ^{3}H-azidopine equally labels both halves of human P-glycoprotein, and vinblastine equally inhibits labelling of the two ^{3}H-azidopine sites within P-glycoprotein [81]. Based on this latter observation, it has been suggested that amino acid residues from the N- and C-terminal halves of P-glycoprotein interact and cooperate to form one major drug interaction pore [81]. However, there is no evidence that this domain defines the only drug-binding site. Rather this region may constitute a three-dimensional structural pocket within P-glycoprotein through which drugs pass during the transport process and from which they are released again. Hence, this model still allows multiple sites for drug recognition and initial drug interactions and is in agreement with the finding that different classes of drugs bind to different, possibly allosterically coupled regions within P-glycoprotein [133, 155, 156]. This model is also supported by the findings that both halves are required for the drug-stimulated ATPase activity of P-glycoprotein [157] and that associations between the two transmembrane domains contribute to interactions between the two halves of P-glycoprotein [63].

Structure–function analyses of naturally occurring and artificially engineered P-glycoprotein mutants have also suggested that both halves of P-glycoprotein are involved in determining its drug substrate and/or MDR modulator specificity and that crucial amino acid residues are generally localised within or near TM regions. The different types of P-glycoprotein mutants analysed include insertion and deletion mutants [158], mutants with one or more single amino acid substitution(s) [159–167], and hybrids between different *MDR* gene products (e.g. mouse *mdr1* and *mdr2* [168, 169], mouse *mdr1* and *mdr3* [170], human *MDR1* and *MDR2* [162, 171]). The hybrid approach is based on the findings that different class 2 *MDR* gene products are unable to function as MDR drug efflux pumps [18, 20] and that different P-

glycoprotein isoforms (e.g human *MDR1* versus mouse *mdr1a* versus mouse *mdr1b*) confer overlapping, but distinct drug resistance phenotypes and show dramatically different responses to MDR modulators [11, 172].

Mutational analyses have provided further evidence for the importance of the TM5-6 and TM11-12 regions for drug interactions. Chinese hamster ovary cells that are highly resistant to actinomycin D allowed the isolation of a P-glycoprotein double mutant carrying an Ala substitution for Gly338 and a Pro substitution for Ala339 in TM6 [163], which subsequently was shown to be impaired in its ability to confer resistance to colchicine and daunorubicin [173]. Substitution of Phe335 in TM6 by Ala or Ser strongly altered the substrate specificity of human P-glycoprotein and impaired its ability to confer resistance to vinblastine or actinomycin D, while the ability to confer resistance to colchicine and doxorubicin was retained [165]. A human P-glycoprotein mutant carrying an Ala substitution for Val338 in TM6 was also affected in its ability to confer resistance to vinblastine, but conferred enhanced resistance to colchicine [166]. In contrast, a Gly341 to Val mutation in TM6 reduced the ability to confer colchicine and doxorubicin resistance, while mutation of Ala342 to Leu in TM6 had a quite general effect and reduced the ability of human P-glycoprotein to confer resistance to colchicine, doxorubicin, vinblastine, as well as actinomycin D [166]. More dramatically, mutation of Ser344 in TM6 to Ala, Thr, Cys, or Tyr completely abrogated the ability of human P-glycoprotein to confer drug resistance [166]. Mutations of Ser941 in TM11 in mouse *mdr1* P-glycoprotein and of Ser939 in TM11 in mouse *mdr3* P-glycoprotein to Phe lowered the capacity to confer colchicine and doxorubicin resistance [161]. These Ser941 to Phe mouse *mdr1* and Ser939 to Phe mouse *mdr3* P-glycoprotein mutants were also less susceptible to inhibition by MDR-reversing agents (verapamil, progesterone and cyclosporin A) and showed reduced binding of ^{3}H-azidopine and ^{125}I-iodoaryl azidoprazosin when compared with the appropriate wild-type P-glycoproteins [174, 175]. A human P-glycoprotein mutant carrying a Phe to Ala or Ser mutation at position 978 in TM12 had lowered capacity to confer resistance to vinblastine and actinomycin D and conferred no detectable resistance to colchicine and doxorubicin [165]. A human *MDR1* mutant carrying *MDR2* substitutions within the TM12 region was found to be poorly labelled with ^{125}I-iodoaryl azidoprazosin and to confer reduced resistance to actinomycin D, vincristine and doxorubicin, but not colchicine [171]. Moreover, an *MDR1* mutant in which the extracytoplasmic loop between TM11 and TM12 was replaced with that of *MDR2* conferred increased resistance for actinomycin D, colchicine and doxorubicin, but not vincristine [171]. Thus, several key amino acid residues are present within the TM5-6 and TM11-12 regions, which when altered, change the drug substrate specificity of P-glycoprotein.

Additional amino acids residues within other membrane-spanning segments or in close proximity thereof may also play a role in determining the cross-resistance pattern conferred by the multidrug transporter. A mutational analysis of thirteen proline residues, five of which are located in putative TM regions, implicated two of these, Pro223 in TM4 and Pro866 in TM10, as being critical for the drug substrate specificity of human P-glycoprotein [164]. The substitution of either of these residues by Ala caused a significant reduction in the ability of human P-glycoprotein to confer resistance to colchicine, doxorubicin and actinomycin D, while vinblastine resistance was increased in the Pro223 to Ala mutant and unchanged in the Pro866 to Ala mutant [164]. Another functionally significant mutation located at position 185 near TM3 in human P-glycoprotein involved a Gly to Val mutation. The *MDR1*-Val185 gene product conferred enhanced resistance to colchicine and etoposide, and reduced resistance to vinblastine, vincristine, actinomycin D, doxorubicin and paclitaxel [142, 159, 160]. Labelling experiments using drug analogues which can be photoactivated indicated decreased colchicine binding and increased vinblastine binding to the *MDR1*-Val185 mutant when compared with wild-type P-glycoprotein, suggesting that a decreased rate of drug dissociation caused reduced vinblastine transport by the *MDR1*-Val185 mutant [142]. Interestingly, the introduction of an Asn183 to Ser mutation near the Gly185 to Val mutation resulted in recovery of resistance to actinomycin D, vinblastine and doxorubicin without a decrease in resistance to colchicine [162]. Other Gly to Val substitutions were made at positions 141 (near TM2), 187 (near TM3), 288 (near TM5), 812 (between TM8 and TM9) and 830 (near TM9), leading to P-glycoprotein mutants which conferred increased resistance to colchicine and doxorubicin [167]. The Gly to Val mutations at positions 187, 288 and 830 also decreased resistance to actinomycin D [167].

Several mutations that alter substrate specificity of P-glycoprotein have also been identified in the N-terminal nucleotide binding fold near the 'Walker B motif'. In a study in which cystic fibrosis-type mutations were introduced at analogous positions into the human *MDR1* gene product, the substitution of Lys536 with Gln slightly decreased resistance to vinblastine and significantly decreased resistance to colchicine and doxorubicin, whereas the substitution with Arg did not affect vinblastine resistance, but increased resistance to colchicine and doxorubicin [176]. In another study in which discrete segments within the N-terminal nucleotide binding fold of the mouse *mdr3* P-glycoprotein were replaced by homologous regions of the C-terminal nucleotide binding fold, residues Glu522, Arg523, Ala525 and Thr578 were found to be crucial for the proper functional activity of the N-terminal nucleotide binding fold [169]. Substitution of these residues by Asp522, Lys523, Thr525 and/or Cys578 profoundly decreased the resistance to colchicine, doxorubicin and actinomycin D, whereas vinblastine resistance was retained [169]. Most of these mutations are located within the 'linker peptide' which has been hypothesised to promote contact of the nucleotide binding fold to the TM domain(s) during the drug transport process [35, 36]. Thus, the stretch from Glu522-Ala525 and residue Thr578 may be directly involved in signal transduction between the nucleotide binding fold(s) and discrete TM domains either upon either binding of drug and/or ATP, or upon ATP hydrolysis [169]. This hypothesis is consistent with the finding that associations between the N-terminal ATP binding fold and the C-terminal TM domain, between the C-terminal ATP binding fold and the N-terminal TM domain, as well as associations between the two ATP binding folds and associations between the two TM domains contribute to interactions between the two halves of P-glycoprotein [63].

Taken together, point mutations that are scattered throughout the primary structure, but reside within or are in close proximity of membrane-spanning segments, contribute to the drug substrate specificity of P-glycoprotein. It remains to be determined which one of these amino acid residues interact

with drug and/or modulator substrate(s) and which ones act indirectly, for example by contributing to conformational changes upon binding of drug and/or ATP, or upon ATP hydrolysis.

ATP INTERACTIONS AND ATPase ACTIVITY OF P-GLYCOPROTEIN

Early cell biology studies indicated that an ATP-driven process was the basis for the multidrug resistance phenomenon since reduced drug uptake and increased drug release in multidrug-resistant cells were sensitive to poisons of mitochondrial respiration (e.g. azide), and reduced intracellular drug accumulation in MDR cells in the presence of azide could be sustained by adding ATP, but not with non-hydrolysable ATP analogues [177]. Subsequent biochemical analyses of drug-selected multidrug-resistant cells or *mdr*-transfectants with analogues of ATP, which can be photoactivated, including ^{32}P-8-azido-ATP [84, 93, 178, 179] and ^{32}P-2-azido-ATP [180], clearly established that P-glycoprotein is an ATP-binding protein. Labelling of P-glycoprotein by ^{32}P-8-azido-ATP was found to be inhibited by an excess of ATP, GTP and the non-hydrolysable ATP-analogues AMP-PNP, but not by ADP, ribose-5-phosphate, or drug substrates [178], suggesting that P-glycoprotein contains specific nucleotide binding region(s) that are different from the drug-binding sites.

As pointed out in the introduction, P-glycoprotein harbours two predicted nucleotide binding folds that are characterised by several conserved elements including the 'Walker A motifs', the 'Walker B motif' [41], the 'centre region', and the 'linker peptide' [42]. Bacterial overexpression, purification and subsequent biochemical characterisation has shown that the C-terminal nucleotide binding fold of P-glycoprotein binds ATP with high affinity [181, 182]. The precise amino acids that are involved in ATP binding have not been identified. Although it has not been directly demonstrated, it is likely that the N-terminal nucleotide binding fold also binds ATP with high affinity, since several studies have indicated that both the N- and C-terminal ATP binding domains are essential for the ATPase and/or drug efflux activity of P-glycoprotein, e.g [56, 58]. Moreover, the N-terminal half of P-glycoprotein has the capacity to catalyse independently ATP hydrolysis [157, 183].

Discrete substitutions of the conserved Gly431 or Gly1073 with Ala, or the conserved Lys432 or Lys1074 with Arg within the 'Walker motif A' in either the N- or C-terminal half of the mouse *mdr1b* gene product abrogated its ability to confer *MDR* [58], suggesting that the two nucleotide binding domains may interact and/or cooperate for P-glycoprotein function. These substitutions, even when introduced in both halves of P-glycoprotein, did not affect labelling of the protein by ^{32}P-8-azido-ATP, implying that a step subsequent to ATP binding in the drug transport process was impaired [58]. Consistent with this idea, similar mutants of the human P-glycoprotein obtained by substituting Lys433 and/or Lys1076 with Met were severely affected in their capacity as drug-stimulated ATPases, although 8-azido-ATP binding was retained [184].

Although both nucleotide binding folds appear to be essential for proper drug transport activity by P-glycoprotein, one unresolved issue has been whether these are functionally equivalent and different approaches have been followed to address this question. The technique of vanadate trapping of Mg- or Co-8-azido-ATP, which non-selectively targets both the N- and C-terminal ATP-binding sites within P-glycoprotein, was used to demonstrate that both the N- and C-terminal nucleotide binding sites are capable of ATP hydrolysis and that vanadate trapping of ATP at either site abolishes ATP hydrolysis at both sites, suggesting that the two sites may alternate in catalysis [185, 186]. Similar conclusions were drawn from a study involving the inactivation by *N*-ethylmaleimide of drug-transporting P-glycoprotein mutants that contain a single Cys in either the N-terminal or C-terminal nucleotide binding fold within the 'Walker A motif' [56].

The drug efflux from MDR cells has been known to be ATP-dependent for a long time, but only recently the high capacity, drug-stimulated ATPase activity of P-glycoprotein has been characterised in detail (reviewed in [187–191]). Initial attempts to purify P-glycoprotein by affinity chromatography resulted in protein preparations with a very low specific ATPase activity (1–3 nmol ATP/min/mg) which could not account for the high consumption of ATP in drug transport assays, using membranes isolated from multidrug-resistant cells [192, 193]. An approximately 1000-fold higher specific ATPase activity (3–5 μmol ATP/min/mg) was reported for recombinant P-glycoprotein in membranes isolated from *MDR1*-baculovirus-infected Sf9 insect cells [71, 84]. Most interestingly, it was also recognised that the P-glycoprotein-specific ATPase activity was stimulated 2–5 fold by different anticancer drugs and chemosensitisers in a dose-dependent manner [84]. Other studies involving membranes of *MDR1*-infected insect cells [194–197], the plasma membrane of Chinese hamster ovary cells selected for high levels of P-glycoprotein expression [180, 198–200], partially purified P-glycoprotein preparations [201, 202] and purified, reconstituted P-glycoprotein [203–207] have corroborated the high capacity ATPase activity of P-glycoprotein which is stimulated by numerous compounds including different anticancer drugs, MDR modulators, anti-oestrogens and steroid hormones, fluorescent calcium- and pH-indicators and bioactive peptides. Initially, it had been assumed that substrates of P-glycoprotein will generally enhance its ATPase activity, but several drugs (e.g. colchicine [203]) have been identified which exhibit very little or no stimulatory effect. Similarly, some MDR modulator substrates (e.g. cyclosporine A) also have no stimulatory effect, but they may interfere with the stimulation of the P-glycoprotein ATPase activity by other compounds [196, 200]. Other MDR modulators (e.g. quercetin) inhibit directly the P-glycoprotein ATPase activity [189]. Moreover, some chemosensitisers (for example progesterone) which are not transported by P-glycoprotein, stimulate its ATPase activity [136, 197, 208], suggesting that they may inhibit P-glycoprotein-mediated drug efflux in multidrug-resistant cells by uncoupling transport from ATP hydrolysis. Thus, the effects of MDR modulators on P-glycoprotein ATPase activity appear diverse and quite complex and a clear explanation for these findings is still outstanding.

Although substantial ATPase activity was measured for P-glycoprotein in all the experimental systems mentioned above, the reported specific ATPase activity varies considerably between different studies. Moreover, the degree of stimulation of ATPase activity elicited by drugs and MDR modulators differs notably and several factors have been suggested to influence the P-glycoprotein ATPase activity directly, including the lipid environment and detergents in the assay [190, 209, 210]. Consistently, it has been found that the P-glycoprotein ATPase activity requires MgATP and is inhibited by

orthovanadate. Several studies have reported on extensive characterisations of the kinetic parameters for the P-glycoprotein ATPase activity and details on substrate, activator and inhibitor specificity (reviewed in [187–191]).

Recently, several investigators have analysed the drug-stimulated ATPase activity of P-glycoprotein mutants with altered substrate specificity [184, 211, 212]. These studies have suggested that often, but not always, the drug-stimulated ATPase activity profile of a P-glycoprotein mutant does correlate with its drug-resistance phenotype [184, 211–213]. Of note also is a study in which P-glycoprotein half-molecules expressed in Sf9 insect cells were found to exhibit ATPase activity, but drug stimulation was only observed when the half-molecules were expressed together, suggesting that interaction between both halves of P-glycoprotein is required for coupling of ATPase activity to drug binding [157].

MODELS FOR THE MECHANISM OF DRUG TRANSPORT BY P-GLYCOPROTEIN

Early drug uptake studies in P-glycoprotein-expressing cells have suggested that decreased drug influx, or increased drug efflux may lead to reduced drug accumulation in MDR cells [177, 214] and the idea has evolved that P-glycoprotein may be responsible for both of these activities [8, 215]. One unresolved issue has been whether P-glycoprotein itself acts as a drug transporter, or whether P-glycoprotein affects drug accumulation in MDR cells via an indirect mechanism. Two major hypotheses for the mechanism of P-glycoprotein have been suggested (reviewed in [6, 8, 189]).

In the first hypothesis it has been proposed that P-glycoprotein functions as an active multidrug transporter that uses energy provided by ATP hydrolysis for the transmembrane translocation of a broad range of structurally unrelated substrates [8]. In the second hypothesis, which was postulated to explain the unusual broad substrate specificity of *mdr* gene products, it has been proposed that P-glycoprotein lowers intracellular drug accumulation indirectly, for example by regulating the plasma membrane pH gradient and/or electrical membrane potential [216–219]. Most of the known P-glycoprotein drug substrates are weakly basic and positively charged at physiological pH, thus an increase of the intracellular pH or a decrease of the negative-internal electrical membrane potential would reduce intracellular retention of these cationic compounds. Experimental data in support of this latter hypothesis include the observations that the pH in the cytosol is often increased [216, 220–223] and that the electrical plasma membrane potential is often reduced in MDR cells when compared with drug-sensitive parental cells [217, 224]. Moreover, it has been demonstrated that changing the intracellular pH can affect the intracellular accumulation of anthracyclins [225]. Conversely, several studies have indicated that decreased drug accumulation in multidrug-resistant cells is independent of alterations of the intracellular pH and/or the plasma membrane potential [226–229]. In an alternative model for an indirect mechanism of drug transport, it has been suggested that P-glycoprotein acts as an outwardly directed ATP channel, thus, generating an electrochemical ATP gradient which drives drugs across the plasma membrane [230]. While indirect mechanisms may contribute to drug resistance in some cases, the proposed models for the P-glycoprotein-associated indirect drug export are difficult to reconcile with the bulk of the experimental data.

The following results support the first hypothesis that P-glycoprotein itself is an active drug transport protein.

(1) Expression levels of P-glycoprotein generally correlate with relative drug resistance of drug-selected cells or *MDR*-transfected cells, expression of a *MDR* cDNA is sufficient to confer the multidrug-resistance phenotype to drug-sensitive cells, and P-glycoprotein is localised at the plasma membrane consistent with its proposed function as a drug efflux pump.

(2) P-glycoprotein itself binds many drug analogues, and single mutations within or near TM segments of P-glycoprotein alter drug binding to P-glycoprotein, therefore modulating its drug substrate specificity.

(3) P-glycoprotein itself binds ATP analogues, exhibits an ATPase activity that is stimulated by a variety of drug substrates, and mutations in either of its predicted ATP binding sites impair its drug efflux activity.

(4) P-glycoprotein belongs to the ABC transport superfamily, shares homology with a variety of prokaryotic and eukaryotic membrane proteins that serve as ATP-dependent transporters for different substrates, and P-glycoprotein can functionally substitute for yeast STE6 as a transporter of the mating factor peptide a [231].

(5) Lower steady-state levels of intracellular drugs are observed in multidrug-resistant cells that differ from drug-sensitive cells only in their expression of P-glycoprotein, and P-glycoprotein-expressing cells are able to extrude daunorubicin against a concentration gradient [232].

(6) ATP hydrolysis-dependent, osmotically sensitive drug transport has been demonstrated in plasma membrane vesicles isolated from P-glycoprotein-expressing cells which is not detectable in plasma membrane vesicles isolated from drug-sensitive parental cells [233–239].

(7) Active transport of P-glycoprotein substrates across the membrane against a significant substrate concentration gradient has been demonstrated by functional expression of mouse P-glycoproteins in yeast secretory vesicles [229]. Moreover, drug transport is not affected by changes in the electrical membrane potential and is independent of a proton gradient and/or proton movements across the membrane [229].

(8) ATP hydrolysis-dependent, concentrative transport of P-glycoprotein substrates has been demonstrated by partially purified P-glycoprotein reconstituted into artificial lipid vesicles [190, 204], and recently by highly purified, reconstituted P-glycoprotein [189, 240]. In other studies ATP-dependent transport of drugs by purified, reconstituted P-glycoprotein has also been described, but it did not occur against a substrate gradient [191, 207]. The elegant experimental system developed by Shapiro and Ling involved a fluorescence-based assay that allowed the transport of the substrate Hoechst 33342, by reconstituted *pgp1* P-glycoprotein, purified from multidrug-resistant Chinese hamster ovary cells, to be continuously monitored [240]. Transport of Hoechst 33342 required hydrolysis of ATP, was dependent on Mg^{2+}, was inhibited by the chemosensitisers verapamil and amidarone, and was blocked by vanadate and N-ethylmaleimide. The reported rate for transport of Hoechst 33342 was very slow (6 nmol/min/mg) and approximately 50 ATP molecules were consumed per molecule of Hoechst 33342 transported, due to an unusually high basal ATPase

activity of the experimental system as well as due to the lack of a trap for the transported substrate allowing futile cycling of the Hoechst 33342 compound between lipid and aqueous phases. Thus, the true rate of transport for the Hoechst 33342 substrate and the real energy requirements of the transport process probably have been obscured and the experimental system may need to be improved to overcome these technical difficulties in future, more detailed analyses.

The demonstration of active drug transport against a substrate concentration gradient with reconstituted, highly purified P-glycoprotein is formal proof that P-glycoprotein itself is capable of the ATP-dependent drug efflux observed in multidrug-resistant cells. However, additional experiments are required to define the exact molecular mechanism of action of P-glycoprotein for which several models have been proposed, including the 'hydrophobic vacuum cleaner' [8] or the 'flippase' [241]. Also, a variety of important questions that have remained unanswered to date will have to be addressed: are most substrates recognised while present within the plasma membrane, or are some exported from the cytosol? What are the kinetics of the drug transport and what is the stoichiometry of ATP hydrolysis and drug transport? Are these parameters substrate dependent? What is the exact mechanism of energy coupling to the drug transport process? Why is the basal ATPase activity of P-glycoprotein relatively high in functional reconstitution systems? Does the lipid composition of the membrane influence ATPase activity and/or drug transport by P-glycoprotein? What are the mechanisms of action of different MDR modulators that block P-glycoprotein-mediated drug transport? How are peptides transported by P-glycoprotein? Does P-glycoprotein have channel activity? Ultimately, the fate of the drug substrates after P-glycoprotein-mediated drug efflux from multidrug-resistant cells will also have to be considered. This may be particularly important with respect to the development of therapeutics to overcome and/or prevent multidrug resistance in human cancer. Do anticancer drugs re-enter the membrane by diffusion? Or are they being trapped, e.g. by plasma binding proteins? Can trapping of effluxed drugs eventually be increased artificially to improve current strategies for clinical reversal of multidrug resistance?

CONCLUSION

Studies and speculations on the physiological role of P-glycoprotein

The efflux of numerous anticancer drugs (Table 1) is clearly a primary activity of class 1 P-glycoproteins in multidrug-resistant cancer cells. However, P-glycoprotein is also found in normal cells where its expression is regulated in a temporal cell- and tissue-specific manner [242–244]. The understanding of the physiological function(s) of P-glycoprotein is important to anticipate the potential side-effects of MDR modulators used to inhibit the function of P-glycoprotein in multidrug-resistant tumours.

Initial ideas on the normal role of P-glycoprotein came from the analysis of the distribution of *MDR* gene products in normal tissues. Such studies have demonstrated that, in human adults, high levels of *MDR1* mRNA are expressed in the adrenal glands, kidney, liver, jejunum, ileum and colon, intermediate levels are expressed in the pregnant uterus and the central nervous system, and low levels are expressed in many other tissues ([245, 246]; reviewed in [247]). In normal mouse tissues, *mdr1b* (*mdr1*) mRNA is predominantly expressed in the adrenal glands, kidney, pregnant uterus and placenta, and *mdr1a* (*mdr3*) mRNA is primarily found in the intestine, testis, liver, lung and brain [248, 249], suggesting that the mouse *mdr1a* and *mdr1b* P-glycoproteins together assume the same or similar physiological function(s) as the human *MDR1* gene product. Immunohistochemical analyses with P-glycoprotein-specific antibodies have revealed that class 1 *mdr* gene products are expressed in a polarised manner on the apical surface of secretory epithelial cell lining lumenal spaces, e.g. the columnar epithelium of the small and large intestine, the brush border of the proximal renal tubule, the biliary canaliculi of hepatocytes, some small pancreatic ductules, the glandular epithelial cells of the endometrium in the pregnant uterus, and in endothelial cells of capillary blood vessels at blood–tissue barrier sites such as brain and testis [243, 248, 250–253]. Moreover, P-glycoproteins have also been detected in CD34 positive bone marrow cells and in circulating lymphocytes ([254], reviewed in [255]).

The presence of P-glycoprotein on the mucosal surface of the small and large intestine and in endothelial cells of capillary blood vessels at blood–tissue barrier sites has suggested that class 1 *MDR* gene products may assume a general protective role and are involved in the defence against xenobiotics ingested with food [250, 252]. Similarly, P-glycoprotein expressed in the kidneys and the liver has been suggested to serve in the excretion of xenobiotics, or possibly endogenous metabolites [250]. Several lines of experimental evidence are in support of these hypotheses: (1) It has been clearly established *in vitro* that P-glycoprotein actively transports a variety of toxic compounds. (2) Mutant mice homozygous for a disruption of the *mdr1a* (*mdr3*) gene exhibit increased sensitivity to the centrally neutrotoxic peptide ivermectin (100-fold) and to the MDR drug vinblastine (3-fold), and show alterations in the pharmacokinetics (a reduced rate of elimination) and tissue distribution (increased accumulation in the brain) of vinblastine infused intravenously [256]. Moreover, the absence of the *mdr1a* P-glycoprotein in these *mdr1a* (-/-) 'knock-out' mice also affects the pharmacokinetics and tissue distribution of other P-glycoprotein substrates, including dexamethasone, digoxin and cyclosporin A [257]; (3) Unidirectional drug transport by endothelial cells from the blood–brain barrier has been demonstrated *in vitro* [258]; (4) Polarised kidney cells that overexpress the human *MDR1* gene product have been shown to secrete digoxin [259]. (5) Circumstantial evidence suggests a role of P-glycoprotein in the secretion of drugs into the bile [260, 261].

Alternatively, the presence of *MDR* gene products on the surface of adrenal cortical cells [250], in the secretory and gestational endometrium of the pregnant uterus [262], and in the trophoblast of the placenta [263] has suggested a possible involvement of P-glycoprotein in steroid transport (e.g. steroid secretion or protection of membranes from toxic effects of dissolved steroids) and evidence in support of this hypothesis has been gained subsequently: (1) Studies of human *MDR1* transfectants have revealed that P-glycoprotein can transport cortisol (a glucocorticoid and the main steroid produced in the human adrenal) [136], aldosterone (the main mineralcorticoid produced in the human adrenal) [136], oestriol (the main oestrogen produced during pregnancy in the fetoplacental unit) [264], and dexamethasone [264]. Both corticosterone and progesterone have been found to interact with mouse P-glycoprotein [265], but do not appear to be a substrate for transport by human P-glycoprotein [264]; (2) P-glycoprotein

inhibitors have been shown to block steroid secretion in mouse adrenal Y1 cells [266]; (3) Steroids have been implicated in the regulation of *MDR* gene expression [253, 267, 268].

The promiscuity of P-glycoprotein as a transporter (Table 1) is astonishing and is in accordance with the idea that P-glycoprotein is an unusual membrane protein which may serve multiple functions. P-glycoprotein expressed in the haematopoietic stem cells has been speculated to modulate their cell differentiation and proliferation via the export of a proposed regulatory molecule [244]. It has also been suggested that P-glycoprotein in lymphocytes may participate in the extrusion of peptides (cytokines?) that lack cleavable hydrophobic signal sequences and are not secreted from cells via the classical signal/cleavage pathway. It is possible that P-glycoprotein shares analogous functional properties with homologous members of the ABC transporter superfamily, for example the Ste6 protein of yeast [269] involved in the export of farnesylated mating factor pheromone a, the haemolysin transport protein HylB of *Escherichia coli* [270], and the endoplasmic reticulum-associated Tap-1 and Tap-2 transport proteins that are involved in antigen presentation [271]. Indeed, P-glycoprotein has been demonstrated to interact with various cyclic and linear peptides of different sizes, e.g. the cytotoxic small peptides gramicidin D and valinomycin, the yeast a-factor pheromone, the calpain and cathepsin inhibitory hydrophobic tripeptide *N*-acetyl-leucyl-leucyl-norleucine, and many other hydrophobic peptides [83, 200, 272, 273]. To date, however, peptide transport by reconstituted, purified P-glycoprotein has not been reported. A functional reconstitution assay would also be invaluable for the evaluation and identification of physiologically relevant peptide substrates of *mdr* gene products.

Finally, it has been suggested that P-glycoprotein may contribute to ion transport and have a physiological role in cell volume regulation. *MDR* gene expression has been associated with the appearance of altered chloride channel activity in response to osmotic shock or nutrient uptake [274–277]. Conversely, it has been reported in several studies that no association between P-glycoprotein expression and cell volume-activated chloride channels is observed [130, 278, 279]. Thus, the nature and physiological role of the P-glycoprotein-chloride channel association has been a subject of debate and still awaits proof of a direct link. Recordings of the P-glycoprotein-associated currents at the single-channel level are technically very demanding experiments and have not yet been reported. Based on the currently available data on P-glycoprotein-associated chloride channel activities, the hypothesis is favoured that P-glycoprotein may act as a chloride channel regulator rather than as a chloride channel itself, but the identity of the regulated chloride channel protein is still obscure (reviewed in [131]). Clearly cells exist which lack detectable P-glycoprotein, but are still able to regulate their cell volume, suggesting that the suggested role for P-glycoprotein in cell volume regulation may be a non-essential one.

In addition to biochemical and cell biological experimental approaches, the generation of mice with ablated *mdr* genes provides the most promising experimental system to learn about the physiological function(s) of P-glycoproteins. Although it is possible that the functions of ablated *mdr* gene products may be partially or fully compensated by related gene products, as suggested by *mdr1a* (-/-) knock-out mice which show increased expression of the *mdr1b* gene product in the liver [256], *mdr1a*-, *mdr1b*- and *mdr2*-defective mice, as well as *mdr1a-mdr1b* double knock-out mice have been highly informative (see Borst and Schinkel, pages 985–990). Studies with mice that are homozygous for a disruption of both the *mdr1a* and *mdr1b* genes are of particular interest from the clinical point of view, as these animals may represent the animal equivalent of human cancer patients during treatment with effective MDR reversing agents that may block the physiological function(s) of P-glycoprotein.

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