Protein kinases and multidrug resistance

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Abstract

The role of protein kinases in the multidrug resistance phenotype of cancer cell lines is discussed with an emphasis on protein kinase C and protein kinase A. Evidence that P-glycoprotein is phosphorylated by these kinases is summarised and the relationship between P-glycoprotein phosphorylation and the multidrug-resistant phenotype discussed. Results showing that protein kinase C, particularly the alpha subspecies, is overexpressed in many MDR cell lines are described: this common but by no means universal finding seems to be drug- and cell line-dependent and in only in a few cases is there a direct correlation between protein kinase C activity and multidrug resistance. From co-immunoprecipitation results it is suggested that P-glycoprotein is a specific protein kinase C receptor, as well as being a substrate. Revertant experiments provide conflicting results as to a direct relationship between expression of P-glycoprotein and protein kinase C. Evidence that protein kinase A influences P-glycoprotein expression at the gene level is well documented and the mechanisms by which this occurs are becoming clarified. Results on the relationship between protein kinase C and multidrug resistance using many inhibitors and phorbol esters are difficult to interpret because such compounds bind to P-glycoprotein. In spite of huge effort, a direct involvement of protein kinase C in regulating multidrug resistance has not yet been firmly established. However, evidence that PKC regulates a Pgp-independent mechanism of drug resistance is accumulating.

Abbreviations: AMP – adenosine-5-monophosphate; DAG – diacylglycerol; EGF – epidermal growth factor; EGFR – epidermal growth factor receptor; LRP – lung resistance-associated protein; MDR – multidrug resistant; MRP – multidrug resistance-associated protein; Pgp – P-glycoprotein; PIP₂ – phosphatidylinositol-4, 5-bisphosphate; PKA – protein kinase A; PKC – protein kinase C; PI – phosphatidylinositol; PLC – phospholipase C; PLD – phospholipase D

Introduction

A major reason for chemotherapy failure in the clinic is that tumour cells may have innate resistance to anti-cancer drugs or may acquire resistance, usually after an initial course of treatment. Colon, gastric and renal cancer cells have innate resistance to a range of chemotherapeutic agents. However, other tumours including breast and ovarian carcinoma and small cell lung carcinoma can acquire resistance and become gradually unresponsive to chemotherapy. The acquisition of this drug resistance is now believed to be due to the establishment of a sub-population of drug-resistant cells from the original tumour which

grow with an acquired resistance to a broad range of structurally-unrelated anticancer agents including anthracyclines, *Vinca* alkaloids, epipodophyllotoxins, antibiotics, colchicine, taxol and others, including small peptides (Bosch and Croop, 1996). This development of a multidrug resistance (MDR) phenotype by tumour cells and the wider area of drug resistance where several mechanisms of resistance may operate, creates a serious difficulty in chemotherapeutic treatment. A full biochemical understanding of such problems is still not available despite the fact that MDR was first described almost twenty years ago and after extensive effort (Roninson, 1991).

A major characteristic of the MDR phenotype is

the overexpression of a multispan plasma membrane glycoprotein, the P-glycoprotein (Pgp), which functions in drug-resistant cells as an ATP-dependent drug efflux pump (Stein, 1997), as discussed in previous chapters. The Pgp molecule has several phosphorylation sites which may be important for regulating its function as discussed below. Pgp is also glycosylated, a feature which may regulate its movement to the plasma membrane rather than influencing its drug efflux function in MDR. It is the purpose of this chapter to review recent results on the role of protein kinases, especially protein kinase C (PKC) and protein kinase A (PKA), in the problem of acquired resistance to anticancer drugs, looking especially at the relationship between PKC and PKA and MDR. The extensive literature on PKC and Pgp has been reviewed recently (for example, Glazer, 1994; Grunicke *et al.*, 1994; O'Brian *et al.*, 1994; Bellamy, 1996; Fine *et al.*, 1996; Gottesman *et al.*, 1996; Srivastava *et al.*, 1996).

Two additional putative drug transporters play some part in MDR and in the wider problem of drug resistance in cancer cells. These are the multidrug resistance-associated protein (MRP) and the lung resistance-associated protein (LRP) described in other chapters in this book. Of these MRP is known to undergo phosphorylation.

Protein Kinases and Protein Phosphorylation

Phosphorylation/dephosphorylation is a common mechanism by which the activity of a protein is regulated and many proteins in signalling pathways, transport processes and metabolism are regulated by this mechanism. Protein kinases phosphorylate suitable hydroxyl groups in a protein substrate and fall mainly into two groups, i) serine/threonine kinases and ii) tyrosine kinases. Protein dephosphorylation is catalysed by protein phosphatases. Phosphorylation of serine/threonine or tyrosine residues can bring about the activation or inhibition of a protein, removal of the phosphate achieving the opposite effect.

The phospholipid-dependent protein kinase C (PKC) and the cyclic AMP-dependent protein kinase A (PKA) are two well-defined kinases which have been implicated in multidrug resistance. PKC phosphorylates serine/threonine residues in the region of basic lysine (K) and arginine (R) residues, for example -S/T-X-K/R-; -K/R-X-S/T-; -K/R-X-X-S/T-; -K/R-X-S/T-X-S/T-K/R- while PKA has a preference for basic residues preceding the S/T phosphorylation site, for

example -R-R-X-S/T-Y- or -R-X-R-X-X-S/T-Y- where Y is a more hydrophobic amino acid residue (Pearson and Kemp, 1991). The Pgp molecule has consensus sites for both PKC and PKA. The relationship between PKC and Pgp has been especially widely examined because PKC activity is upregulated in many cancer cell lines which also show increased expression of Pgp (see Section 3.2 below).

Protein Kinase C and Multidrug Resistance

Protein kinase C

The protein kinase C (PKC) family of structurally-related phospholipid-dependent serine/threonine kinases (Dekker *et al.*, 1995) are involved in signal transduction pathways regulating a wide range of key biological events (Hug and Sarre, 1993; Kuo, 1994; Stabel, 1994; Buchner, 1995; Pears, 1995; Newton, 1997) and many substrates for PKC have been identified (Liu, 1996). The finding that PKC was activated by diacylglycerol (DAG) highlighted the importance of the kinase in growth factor- and hormone-activated signal transduction pathways where DAG is generated by hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP₂) by the action of a PI-specific phospholipase C (PI-PLC). Pathways involved in the transient and sustained formation of DAG for PKC activation have been reviewed by Nishizuka (1995). PKC is a major receptor in cells for the tumour-promoting phorbol esters (Rando and Kishi, 1992) and such molecules are now widely used as DAG analogues to activate PKC. Unlike DAG, phorbol esters are not readily metabolised and so induce long term activation of PKC and a sustained response in cells. This makes it easier to observe the effects of PKC activation but such prolonged responses are probably non-biological and lead to rapid downregulation of susceptible PKC subspecies by proteolysis making results from such experiments very difficult to interpret.

PKC subspecies

The PKC family now consists of eleven subspecies which are divided into three main classes on the basis of domain homology and their activation requirements: the conventional subspecies, α , β_I , β_{II} and γ require diacylglycerol and calcium for full activity, the novel subspecies, δ , ϵ , η , θ lack the requirement for calcium while the atypical subspecies ζ and ι (mouse

λ) are active independently of diacylglycerol or calcium. Only conventional and novel PKC subspecies are activated by phorbol esters. PKC- μ has a requirement for DAG, but not calcium ions, for activation so can be regarded as a novel PKC subspecies; it is often grouped separately since it has a kinase domain which is more closely related to calmodulin-dependent kinases than other PKCs (Dekker *et al.*, 1995).

PKC structure

The structure of PKC, with details of conserved and variable polypeptide regions in each subspecies, of the C-terminal kinase domain and the N-terminal regulatory domain and the structural interrelationships between subspecies, have been extensively summarised (Hug and Sarre, 1993; Newton, 1995, 1997; Pears, 1995; Hofmann, 1997). The relationship between PKC and related gene families such as the newly discovered PKC-related kinases (PRKs) has been reviewed by Dekker *et al.* (1995). Various studies have led to the identification of an intramolecular inhibitory domain in the regulatory region of PKC subspecies. This 'pseudosubstrate site' lacks a serine or threonine and thus cannot be phosphorylated. This site, however, brings about inhibition of the kinase catalytic site in a PKC molecule which is postulated to be bent back on itself at the V3 hinge region into a hairpin shape (Newton, 1995; Pears, 1995). Activation of a PKC molecule by interaction of the regulatory region with DAG and phospholipid in a membrane site is thought to release the kinase domain from inhibition by the pseudosubstrate peptide sequence leading to an active catalytic site and at the same time making the molecule sensitive to proteolytic turnover by cleavage at the V3 region (Dekker *et al.*, 1995; Pears, 1995; Hofmann, 1997). Use of inhibitor peptides based on the pseudosubstrate site are proving useful in quantitative assays of PKC activity (e.g. Drew *et al.*, 1994) and have also been used to try and inhibit PKC subspecies in *in vivo* experiments in free and myristoylated forms (Gupta *et al.* 1996).

PKC phosphorylation

New results suggest that PKC has to be phosphorylated by an as yet undefined PKC kinase before it can autophosphorylate and become catalytically active. Initial phosphorylation of Thr 497 in PKC- α , or Thr 500 in PKC- β_{II} in an activation loop region is essential for further PKC phosphorylation at two other sites. Phosphorylation at Thr-638 in PKC- α controls dephosphorylation and inactivation of the ki-

nase. Current findings on PKC phosphorylation and regulation are summarised by Newton (1995, 1997). This author also gives a model for PKC activation in which newly-synthesised 'nascent' PKC- β_{II} associates with a detergent-insoluble cytoskeleton fraction in cells where it is phosphorylated on Thr-500 by the putative PKC-kinase. This transphosphorylation allows autophosphorylation to take place on residues Thr-641 and Ser-660 at the C-terminus releasing the protein. Phosphorylated PKC then migrates to an anchoring protein site at a membrane where release of pseudosubstrate site inhibition and attainment of catalytic activity can then take place on interaction with DAG or phorbol esters (Newton, 1995, 1997; Pears, 1995). A leading question must now relate to the nature of the putative PKC kinase and what initiates its activation to catalyse the first transphosphorylation.

PKC subspecies expression

Several PKC subspecies are expressed together in cells suggesting that individual PKCs have discrete, non-overlapping, functions. Alternatively, it might simply be that the range of subspecies provide surplus capacity for phosphorylation reactions. The latter view is now considered unlikely considering the structural differences between subspecies, their different activation requirements, the finding that protein expression of individual subspecies changes with cell growth status and the fact that individual subspecies translocate to different intracellular sites on activation. Further, different signal transduction pathways may also bring about activation of specific PKC subspecies. For example, the lipid product of PI 3-kinase activation, PtdIns-3,4,5-P₃, can effectively activate PKC- ζ but not conventional subspecies (Nakanishi *et al.*, 1993) while ceramide generated in the sphingomyelin cycle by a sphingomyelinase (Hannun, 1994), specifically causes translocation of PKCs- δ and - ϵ from the membrane to the cytosol (Sawai *et al.*, 1997) and can inhibit PKC- α (Lee *et al.*, 1996). Some agonists including bombesin, endothelin-1 and platelet-derived growth factor, for example, stimulate signaling pathways leading to the selective activation of PKCs- δ and/or - ϵ (Kiley *et al.*, 1995) again suggesting that individual PKCs have distinct functions in a cell. However, demonstrating this is so *in vivo* is proving more difficult (Hug and Sarre, 1993; Dekker and Parker, 1994; Liu, 1996).

Targeting PKC in cells

Anchoring and scaffold proteins are involved in the targeting of PKC subspecies to, and their interaction with, a specific substrate. It is suggested that inactive PKC subspecies are released from the cytoskeleton after phosphorylation and are then targeted to subcellular sites through interactions with other proteins, several of which have been identified (Kiley *et al.*, 1995; Newton, 1996). These targeting proteins may be specific substrates for PKC (adducin, MARCKS, PICKS 1 & 2), may contain interaction domains, may be receptors for activated PKC (RACKs) or may even be complex scaffold proteins such as AKAP 79 which binds PKA and the phosphatase calcineurin, as well as PKC (Klauck *et al.*, 1996). Many of these target proteins bind phosphatidylserine (PS) which is needed for target protein-PKC interaction. Discrete amino acid sequences on PKC, important for interaction with target proteins, have been identified in both regulatory and catalytic domains, in conserved regions of the molecules and in sites specific to individual PKC subspecies. Newton (1996) has provided a succinct summary of knowledge on this topic and such new ideas must be now related to the interaction of PKC subspecies with substrates such as Pgp in cells showing the MDR phenotype (see section below).

PKC downregulation

Long term phorbol ester activation of some conventional and novel PKC subspecies results in their gradual loss from a cell. This process, down-regulation, is due to an increased rate of proteolysis with no effect on synthesis (Parker *et al.*, 1995). The enzyme responsible for cleaving the PKCs initially at the V3 hinge region, probably a calcium-dependent protease (calpain), has not been fully defined. The kinase fragment released by such proteolysis (PKM) retains catalytic activity. Atypical PKCs are not sensitive to phorbol esters so do not down-regulate though phorbol ester-treatment can result in translocation of PKC- ζ (Dang *et al.*, 1994). The down-regulation rate of individual PKC subspecies varies considerably. In TPA-stimulated fibroblasts Olivier and Parker (1992) showed that PKCs α , β and δ are down-regulated within 6–12 hr while PKC- ϵ is lost much more slowly. An increase in endocytic vesicle traffic may play an important role in the mechanism of PKC subspecies down-regulation (Goode *et al.*, 1995; Parker *et al.*, 1995). DAG activation of PKCs also induces proteolytic turnover of individual PKC subspecies (Olivier and Parker, 1994).

A further view of PKC turnover has come from the recent identification of ubiquitinated forms of PKC- α and perhaps also of PKC- ϵ (Lee *et al.*, 1996, 1997). These were detected in Bryostatine-treated cells as higher molecular weight forms of the PKCs which accumulated when the proteasome inhibitor lactacystin was added. The 26 S proteasome is a non-lysosomal site of proteolysis located in the nucleus and cytosol which may play a role in the normal turnover of some PKC subspecies compared with the possibly artificial down regulation process induced by phorbol ester treatment of cells.

PKC activity in MDR cell lines

Many, if not most, MDR cell lines have elevated levels of PKC activity and increased protein expression, often by considerable amounts. For example, total cytosolic PKC activity is increased four-fold in doxorubicin (Adriamycin)-selected MDR KB-A1 cells compared with the parental drug-sensitive KB-3-1 line (Drew *et al.*, 1994). Such findings are summarised in O'Brian *et al.* (1994) and in Table I. In general, it seems that the increased level of PKC activity and protein expression depends on intrinsic features of the parental cell line and/or the drug used for selecting the MDR cell population. In two drug-resistant human KB carcinoma cell lines we found that while KB-V1 cells had a higher Pgp level than KB-A1 cells, total cytosolic and membrane PKC activity was in the reverse order KB-A1 > KB-V1 (Drew *et al.*, 1994). In general, the level of PKC activity in total cell extracts of doxorubicin-selected MDR cell lines is higher than in the drug-sensitive cells from which they were derived. For MCF-7/DOX^R, S180A10 and KB-A10 cells, PKC activity was increased in both cytosolic and membrane fractions relative to the drug-sensitive line (Posada *et al.*, 1989a). We observed the same for a series of MDR KB cells (Drew *et al.*, 1994) though for KB-A1 cells we found a 5 fold increase in membrane-associated PKC and no increase in KB-8-5 cells compared with the 11 fold and 5 fold increases respectively in the same cell lines reported by Hu and Robert (1997) – see Table I. Such differences almost certainly arise from the different assay methods used. With MCF-7/ADR cells, an increase in PKC activity has also been observed in a nuclear fraction (Lee *et al.*, 1992). Furthermore, *Vinca* alkaloid-selected MDR cell lines show increased PKC activity although levels in total cell extracts of only two such cell lines, KB-V1 and DC-3F/VCRd-5L, have been reported (Chambers *et*

Table 1. Comparison of total, cytosolic and membrane associated protein kinase C activity in some drug-sensitive and drug-resistant MDR cell lines^a

Cell line	Phenotype	Total PKC	Cytosol PKC	Membrane PKC	Selecting agent
UV-2237M	DS	1.0	–	–	–
UV-2237M-rev	MDR	1.52	–	–	Doxorubicin
UV-2237M-ADR ^R	MDR	2.4	–	–	Doxorubicin
UV-2237M-ADRR ^R	MDR	1.84	–	–	Doxorubicin
O'Brian <i>et al.</i> , 1989					
MCF-7 WT	DS	1.0	–	–	
MCF-7/DOX ^R	MDR	7.02	–	–	Doxorubicin
Fine <i>et al.</i> , 1988					
MCF-7/S	DS	1.0	–	–	
MCF-7/R	MDR	15.0	–	–	Doxorubicin
Palayoor <i>et al.</i> , 1987					
MCF-7/WT	DS	1.0	1.0	1.0	
MCF-7/DOX ^R	MDR	6.1	5.0	8.8	Doxorubicin
Schwartz <i>et al.</i> , 1991					
HL-60/S	DS	1.0	–	–	
HL-60/R	ADR-res	1.6	–	–	Doxorubicin
Palayoor <i>et al.</i> , 1987					
HL-60	DS	–	1.0	–	
HL-60/ADR	ADR-res	–	1.5	–	Doxorubicin
Aquino <i>et al.</i> , 1988					
P388/S	DS	1.0	–	–	
P388/R	MDR	1.1	–	–	Doxorubicin
Palayoor <i>et al.</i> , 1987					
P388	DS	1.0	1.0	1.0	
P388/VP-16	MDR	0.67	0.83	0.58	VP-16-213
Ido <i>et al.</i> , 1987					
S180	DS	–	1.0	1.0	
S180-A10	MDR	–	1.77	1.13	Doxorubicin
Posada <i>et al.</i> , 1989a					
KB-3-1	DS	–	1.0	1.0	
KB-A10	MDR	–	1.7	9.0	Doxorubicin
Posada <i>et al.</i> , 1989a					
KB-3-1	DS	1.0	–	–	
KB-V1	MDR	4.1	–	–	Vinblastine
Chambers <i>et al.</i> , 1990a					
KB-3-1	DS	–	1.0	–	
KB-A1	MDR	–	5.0	–	Doxorubicin
KB-A10	MDR	–	5.0	–	Doxorubicin
Dolci <i>et al.</i> , 1993					

Table 1. (continued)

Cell line	Phenotype	Total PKC	Cytosol PKC	Membrane PKC	Selecting agent
KB-3-1	DS	—	1.0	1.0	
KB-A1	MDR	—	4.2	5.2	Doxorubicin
KB-V1	MDR	—	1.6	2.2	Vinblastine
KB-C1	MDR	—	3.4	3.4	Colchicine
KB-8-5	MDR	—	1.1	1.0	Colchicine
KB-8-5-11	MDR	—	1.8	2.8	Colchicine
Drew <i>et al.</i> , 1994					
KB-3-1	DS	—	—	1.0	
KB-8-5	MDR	—	—	5.6	Colchicine
KB-A1	MDR	—	—	11.3	Doxorubicin
Hu and Robert, 1997					
C6S	DS	—	—	1.0	
C6 IV	MDR	—	—	0.17	Vincristine
C6 0.5	MDR	—	—	0.99	Doxorubicin
Hu and Robert, 1997					
DC-3F	DS	1.0	—	—	
DC-3F/VCRd-5L	MDR	1.5	—	—	Vincristine
Palayoor <i>et al.</i> , 1987					
MOLT3	DS	1.0	1.0	1.0	
MOLT3/TMQ200	MDR	0.59	0.47	0.55	Trimetrexate
MOLT3/TMQ800	MDR	0.46	0.46	0.46	Trimetrexate
MOLT3/TMQ2500	MDR	0.51	0.65	0.40	Trimetrexate
Schwartz <i>et al.</i> , 1991					

^a Figures represent relative values of PKC activity normalised to 1.0 in the drug-sensitive line in each case. DS = drug sensitive; MDR = drug resistant.

al., 1990a; Palayoor *et al.*, 1987). There are exceptions to such results, however. In a few cases, PKC activity in drug-resistant cells may be unchanged, as has been reported in a vincristine-selected P388 MDR cell line (Ido *et al.*, 1986), or even decreased as in three MDR MOLT3 cell lines selected with the antifolate trimetrexate, an epipodophyllotoxin-selected P388 MDR cell line or MDR C6 glioma cells (Ido *et al.*, 1987; Schwartz *et al.*, 1991; Hu and Robert, 1997). MDR cell lines thus mostly show increased PKC activity but there are sufficient exceptions to show that this is not a universal feature of multidrug resistance.

Changes in both conventional and novel/atypical PKC activities have been assayed in a limited number of MDR cell lines. In MCF-7/ADR cells a 10-fold increase in cytosolic calcium-dependent activity and a 10-fold decrease in cytosolic calcium-independent activity compared to the drug-sensitive parental line

has been reported (Blobe *et al.*, 1993). Cytosolic PKC activity was reduced in the weakly resistant AH66DR-0.3 line although it was increased in the more highly resistant AH66DR-30 line compared with the parental line. This elevated PKC activity was mainly either calcium-independent/phospholipid-dependent or calcium-independent/phospholipid-independent (Ohkawa *et al.*, 1994a). In addition, transiently increased PKC activity observed in AH66DR cells following doxorubicin treatment was reported to be mainly either calcium-independent/phospholipid-dependent or calcium-independent/phospholipid-independent (Ohkawa *et al.*, 1994b). We found that calcium-independent PKC activity was increased in a series of MDR KB cells compared with drug sensitive KB-3-1 cells especially in the membrane compartment (Drew *et al.*, 1994).

The studies outlined above, and summarised in Table I, have led to the view that the multidrug resistance phenotype is often associated with increased levels of PKC activity and that PKC and MDR must therefore be related in some way. This finding is to some extent reinforced by some observations showing that the level of PKC activity correlates with the degree of multidrug resistance to a specific drug (doxorubicin) as has been reported in a series of UV-2237M cell lines (O'Brian *et al.*, 1989). We found the same in a series of KB cells selected in colchicine (Drew *et al.* 1994). Here, apart from the weakly resistant KB-8-5 line which showed the same PKC activity as parental drug sensitive KB-3-1 cells (though see Hu and Roberts, 1997, Table I), total PKC activity in cytosol and membrane increased with increasing MDR status. A correlation of calcium-independent PKC activity with increasing MDR in cytosol and membrane compartments of these cells was not apparent (Drew *et al.*, 1994). However, it should be stressed that this relationship did not hold with KB cells made resistant to different drugs, as mentioned above. There are other exceptions. For example, KB-A1 and -A10 cells which are 100- and 1000-fold resistant to Adriamycin have similar levels of cytosolic PKC compared with the drug sensitive line though, unusually, expression of Pgp in the more resistant A10 line is lower than in the A1 line (Dolci *et al.*, 1993). In addition, the studies of Ido *et al.* (1987), Schwartz *et al.* (1991) and Hu and Roberts (1997) summarised in Table I and mentioned above indicate that the MDR phenotype is not always associated with increased levels of PKC.

PKC activity can also be altered in MDR cells which do not express Pgp. For example, PKC activity is increased 2–3 fold in MDR HL-60/ADR cells which do not express Pgp (Aquino *et al.*, 1990): this was found to be due to induced expression of PKC- γ which is not expressed in the parental form. Richon *et al.* (1991) also detected elevated PKC- ϵ protein in a subline of Friend erythroleukaemia cells which had a low level of resistance to vincristine.

Altered expression of PKC subspecies in MDR cells

Where PKC activity is increased in MDR cells it is commonly, though not exclusively, the α -subspecies which is present at higher protein, and often message, levels. We observed this in a detailed examination of PKC subspecies expression in a series of drug-resistant KB cell lines (Drew *et al.*, 1994). PKC- α was the only subspecies to be consistently elevated in pro-

tein levels in all drug-resistant KB cells compared with the drug-sensitive parental KB-3-1 line as found more recently by Cloud-Heflin *et al.* (1996) and Hu and Roberts (1997). These latter workers also found that KB-A1 cells overexpress PKC- δ in agreement with our findings though we found no evidence that either KB-3-1 or KB-A1 cells overexpressed PKC- γ protein as Hu and Robert (1997) have reported. Protein levels of PKC- α were increased over six fold in doxorubicin-selected KB-A1 cells and over three fold in colchicine-selected KB-C1 cells. The increase in PKC- α protein in KB-A1 cells was membrane-associated as well as cytosolic. The only other subspecies with increased protein expression was PKC- δ in KB-A1 cells and this was largely membrane-associated (Drew *et al.*, 1994) as is the case in the parental KB-3-1 line. Others have noted reciprocal changes in PKC subspecies in MDR cells: PKCs δ and ϵ decreased in protein expression in MDR MCF-7 cells compared with the parental line while both mRNA and PKC- α protein were observed (Blobe *et al.*, 1993). Davies *et al.* (1996) have recently separated two populations of drug-resistant MCF-7/Dox cells which differ in their degree of Pgp expression and mRNA for Pgp. High and low Pgp cell populations showed the same PKC subspecies profile in which expression of the α and θ subspecies was greatly elevated compared with drug-sensitive MCF-7 cells while expression of PKCs ϵ and ζ was reduced. The question of which PKC subspecies is increased in the multidrug resistance phenotype appears to depend on the cell line involved and the drug producing resistance. We note, however, in our KB cell studies that development of resistance to colchicine, doxorubicin or vinblastine was always associated with increased PKC- α protein. Cytosol and membrane levels of PKC activity did not correlate with resistance, i.e. PKC activity and PKC- α protein expression was highest in KB-A1 cells which had a lower relative resistance than KB-V1 or KB-C1 cells.

It is still not clear why PKC- α among the range of subspecies should be selected in this way or how this selection occurs. Discrepancies in the literature regarding which PKC subspecies are expressed in the same drug-sensitive and drug-resistant lines may be the result of using different antibodies, practical techniques, etc. (Blobe *et al.*, 1993/Yang *et al.*, 1996; Drew *et al.*, 1994/Hu and Roberts, 1997) do not help to clarify the picture. However, generally, PKC- α is the main subspecies which shows altered expression in drug-resistant cells where PKC activity is increased. Interestingly, PKC- α is the subspecies which is el-

evated in the intrinsically-resistant LoVoC1.7 cells which have never been exposed to anti-cancer drugs (Dolfini *et al.*, 1993). On the other hand, the increased PKC activity in MDR HL-60/ADR cells which do not express Pgp (Aquino *et al.*, 1990) was due to induced expression of PKC- γ which is not expressed in the parental form while Richon *et al.* (1991) detected elevated PKC- ϵ protein in a subline of Friend erythroleukaemia cells which had a low level of resistance to vincristine. In P388/ADR cells PKC- β may play a role in the MDR phenotype (Gollapudi *et al.*, 1995); introduction of an anti-PKC- β antibody, but not an anti-PKC- α antibody corrected the daunomycin accumulation defect and reversed resistance to daunorubicin.

More recently the relationship between PKC and Pgp in MDR has been explored with revertant cell lines produced by growing resistant cells in the absence of drug for many passages until Pgp expression is markedly decreased or lost altogether. Revertant cells produced from the KB-V1 MDR line showed no Pgp expression and PKC levels were also reduced to below those of the drug-sensitive parental KB-3-1 cells (Cloud-Heflin *et al.*, 1996). The same was found with revertant MCF-7/ADR cells where again Pgp and PKC expression decreased to parental cell levels (Budworth *et al.*, 1997). A revertant KB-C1 cell line showing no Pgp expression also had markedly reduced PKC- α subspecies protein expression compared with the original MDR KB-C1 line (Drew, 1996). Such findings might add strength to the view that expression of PKC and Pgp in MDR are linked for some functional reason. In contrast, however, we (Fenton, 1996) have produced revertant cells from the most resistant cell line in a series of vincristine-resistant CHO cells (Warr *et al.*, 1988; Stow and Warr, 1991). After several months of growth and passage in drug-free medium Pgp expression was decreased to barely detectable levels but, in three different cell clones, PKC expression was the same, if not higher, than in the original resistant line. This finding suggests that Pgp and PKC expression is not linked but, with the other findings from the same approach, shows yet again that the relationship between PKC and Pgp expression may differ from cell type to cell type and depend on the selecting drug.

The elevated levels of PKC activity and of PKC- α protein shown by MDR cell lines are often described as resulting from overexpression of the protein and in some cases this may be correct. For example, KB-A10 cells have increased mRNA for PKC- α while mRNA

for both PKCs α and β is elevated in P388/ADR cells (Posada *et al.*, 1989a; Gupta *et al.*, 1994a). However, this is not observed in S180A10 cells which also overexpress PKC- α : it is also not found in the intrinsically-resistant LoVoC1.7 cell line which has not been exposed to chemotherapeutic agents (Dolfini *et al.*, 1993). Increased message stability and/or increased translation could account for increased PKC subspecies expression in such cases, as has been reported for P388/ADR cells which have decreased mRNA but increased PKC- δ , - ϵ and - ζ protein levels compared to drug-sensitive P388 cells (Gupta *et al.*, 1994a).

An increased level of PKC protein in such cells would also be observed if the rate of proteolytic turnover of the kinase was decreased. There is already some evidence that decreased turnover may account for the elevated PKC levels observed in MDR UV-2237M cells selected for doxorubicin (Ward and O'Brian, 1991). These cells showed altered phorbol ester-mediated downregulation. Cloud-Heflin *et al.* (1996) have shown the same feature in KB-V1 and KB-A1 drug-resistant cells where defective phorbol ester-mediated turnover of PKC- α was observed compared with drug-sensitive KB-3-1 cells. Drug-resistant and drug-sensitive cell lines showed the same cellular uptake of phorbol ester and also expressed similar levels of a PKC-binding protein RACK1. An involvement of RACK1 in multidrug resistance has not been established. Such studies suggest that defective PKC proteolysis may account for the elevated levels of PKC activity and of PKC- α subspecies protein observed in MDR cell lines. This aspect needs to be examined urgently in relation to new findings on the involvement of vesicle traffic in PKC degradation and especially on ubiquitinylation and proteasome involvement in PKC- α turnover (Lee *et al.*, 1996, 1997). MDR cells are known to have altered rates of vesicle movement and increased membrane recycling (Simon and Schindler, 1994). Such changes may influence rates of PKC turnover. It is important that mechanisms regulating the elevated levels of PKC subspecies in cancer cell lines, and especially in primary cancer cells, be established since they may represent a target for therapy (Basu, 1993).

Pgp phosphorylation by PKC

Pgp is a phosphoprotein *in vivo* and has over 40 consensus sites for phosphorylation by kinases like PKC and PKA (Germann *et al.*, 1995). It has proved rela-

tively straightforward to show that, of these possible residues, serines 661, 667 and 671 in the 60 amino acid linker region joining the two halves of the human Pgp molecule are the main sites phosphorylated by PKC in human Pgp, at least *in vitro*. Such findings have been made using synthetic peptides of the linker region (Chambers *et al.*, 1994), on Pgp in membrane preparations from drug-resistant cells incubated with purified PKC (Chambers *et al.*, 1993) and on intact cells stimulated with phorbol ester to activate PKC (Chambers *et al.*, 1990a,b, 1992). The same situation pertains in murine Pgp and involves serines 665, 669 and 681 in the linker region (Orr *et al.*, 1993; Glavy *et al.*, 1997). Pgp undergoes rapid phosphorylation and dephosphorylation (Ma *et al.*, 1991), protein phosphatases catalysing the reverse reaction (Chambers *et al.*, 1992). Key literature on Pgp phosphorylation and its role in MDR has been comprehensively reviewed by Germann *et al.* (1995) and by Fine *et al.* (1996). It is proving less straightforward to discover what effect phosphorylation has on Pgp function.

The specific PKC subspecies involved in Pgp phosphorylation *in vivo* is not known but overexpression of PKC- α in BC-19 cells resulted in increased phosphorylation of Pgp (Yu *et al.*, 1991; Ahmad *et al.*, 1992) while antisense cDNA to this subspecies transfected into MCF-7/ADR cells reduced Pgp phosphorylation. On the other hand PKC- γ transfected into BC-19 cells had no effect on Pgp phosphorylation suggesting that Pgp is not an endogenous substrate for PKC- γ in these cells (Ahmad *et al.*, 1992). PKC- α phosphorylates Pgp *in vitro* when co-expressed in a baculovirus system (Ahmad *et al.*, 1994). This study was undertaken with isolated membrane vesicles and evidence of PKC autophosphorylation which could be blocked with Ro31-8220 was also observed. In the light of what is now postulated for PKC activation (see earlier) this autophosphorylation may also include the action of the unknown PKC-kinase which catalyses the initial phosphorylation of the PKC- α prior to autophosphorylation and full activation. This work also reveals that Pgp and PKC- α must be closely associated in cell membranes since they can be co-immunoprecipitated with either antibody. Yang *et al.* (1996) found the same in both MCF-7/AdrR and KB-V1 cell lines raising the possibility that Pgp functions as a specific receptor for PKC in MDR cells though this is probably not PKC- α specific since Yang *et al.* (1996) also found that the β , γ , ϵ and θ subspecies, but not PKCs- δ , $-\mu$, $-\zeta$ or $-\lambda$, were co-immunoprecipitated with Pgp from MDR cells with either antibody.

In vitro, however, most PKC subspecies including PKCs βI , βII , γ , δ , ϵ , η and ζ can phosphorylate Pgp with differing degrees of efficiency, PKC- βI being the most effective (Fine *et al.*, 1996). There is some evidence that individual PKC subspecies phosphorylate Pgp at different sites (Sachs *et al.*, 1995a) while phosphorylation by different PKC subspecies can have bidirectional effects on the *Vinca* alkaloid-stimulated ATPase activity of Pgp (Fine *et al.*, 1996). In spite of all such results however, it is still not clear which PKC subspecies phosphorylate Pgp naturally in MDR cells even though PKC- α is the subspecies most usually overexpressed. In P388/ADR cells anti-PKC- β but not anti-PKC- α antibody reversed resistance to daunomycin (Gollapudi *et al.*, 1995) suggesting a role for PKC- β in the MDR phenotype of such cells. Again, the PKC subspecies involved in Pgp phosphorylation may be cell-type and drug specific.

The PKC-anchoring protein RACK (Mochly-Rosen, 1995) was detected by western blotting in both drug-sensitive and drug-resistant MCF-7 and KB cells (Yang *et al.*, 1996) but the RACK protein was not detected in PKC-Pgp co-immunoprecipitates. Further, RACK protein expression was the same in non-Pgp- and Pgp-expressing KB and MCF-7 cells. This suggests that RACKS are not involved in targeting PKC subspecies to Pgp. The co-immunoprecipitation studies do suggest, however, that PKC and Pgp must be closely associated in membranes of drug-resistant cells. Pgp in MDR cells should perhaps, therefore, be included with adducin and the perinuclear protein PICK1 as a PKC-binding protein which is also a direct substrate (Newton, 1996). Such studies show that there is a close association between PKC and Pgp in drug-resistant cells but do not provide an understanding as to the function of Pgp phosphorylation.

A positive way to examine the role of Pgp phosphorylation by PKC in MDR would be to block the ability of PKC to phosphorylate the relevant serine residues in the linker region. This could be achieved with inhibitors of PKC but many such inhibitors are substrates for the drug efflux activity of Pgp to confuse results (see below). The alternative, to remove relevant serine residues from Pgp to prevent their phosphorylation and to examine the drug binding and efflux properties of Pgp mutated in this way, has now been achieved (Germann *et al.*, 1996; Goodfellow *et al.*, 1996). Mutant human Pgps with PKC- and PKA-directed serine residues in the linker region changed to alanine, or to aspartate/glutamate to provide negative charge, have been expressed in fibroblasts or

KB-3-1 carcinoma cells. The mutant Pgps conferred MDR characteristics on the drug sensitive cells, had similar expression and subcellular localisation characteristics as wild-type Pgp while drug transport properties were the same. These studies also reveal that PKC does not phosphorylate Pgp outside the linker region since incubation of the mutated forms of Pgp with PKC or in cells showed no evidence of significant phosphorylation elsewhere in the molecule. The general conclusions from such studies are that phosphorylation (and dephosphorylation) are not essential for basal drug efflux activity of Pgp and development of the MDR phenotype. This was also implied by a preliminary study of Buschman and Gros (1991) using a chimeric *mdr1* Pgp containing the *mdr2* linker region which lacks -R-R-X-S- phosphorylation sites. The mutant Pgp forms described above can be used to answer further questions, for example, whether similar findings occur when they are transfected into other cell types, if the turnover time of mutant Pgp is similar to the normal form and how phosphorylation of individual serine residues in the linker region influences drug transport.

Nevertheless, Pgp is clearly phosphorylated *in vivo* and activation of PKC in some cell types increases Pgp phosphorylation and drug transport activity. So how do such findings square with the mutant Pgp results described above? Immunoprecipitation results clearly show that PKC and Pgp interact closely in the membrane and, as suggested by Bates *et al.* (1992) and implied more recently by Germann *et al.* (1996), phosphorylation of Pgp may influence drug substrate specificity and drug binding specificity. It may also regulate the ATPase activity of Pgp (Ahmad *et al.*, 1994; Fine *et al.*, 1996) perhaps accounting for the increased drug resistance seen in MDR cells which overexpress PKC. It may also be that phosphorylation by PKC has more to do with Pgp structural organisation or its normal functions (Stein, 1997) which are distinct from its role in MDR.

Recent findings by Glavy *et al.* (1997) that murine Pgp is phosphorylated *in vivo* in the acidic domain of the linker region, where five potential serine phosphorylation sites exist, provides a new focus for extending work on the relevance of phosphorylation to Pgp function in the MDR phenotype. However, this finding somewhat conflicts with the mutation results above where no evidence of significant *in vivo* phosphorylation at other sites was detected in mutant Pgp lacking PKC sites in the linker region and transfected into mouse fibroblasts or human KB-3-1 carcinoma cells.

PKC inhibitors and MDR

The role of PKC in the MDR phenotype has been widely studied using a range of pharmacological agents known to inhibit PKC. Generally such inhibitors have been found to decrease levels of drug resistance to MDR-associated drugs in a variety of Pgp expressing cell lines and such effects are often associated with enhanced drug accumulation and reduced drug efflux. There are, however, serious problems associated with the use of many such inhibitors which are not always appreciated. In particular, staurosporine and many of its derivatives which inhibit PKC by binding at the catalytic kinase site are not PKC-specific (Gordge and Ryves, 1994). Thus effects observed may be due to the inhibition of kinases besides PKC, perhaps even the unknown PKC kinase which initially activates PKC (section 3.1.3 above). Another serious problem with many kinase inhibitors is their ability to bind to Pgp inhibiting Pgp-mediated drug transport by competing with drug substrates (Smith and Zilfou, 1995). In *in vitro* studies staurosporine, many staurosporine derivatives, isoquinoline sulphonamides, calphostin C and NPC 15437 have all been found to inhibit photoaffinity labelling of Pgp by the calcium channel blocker, [³H] azidopine (Sato *et al.*, 1990; Wakusawa *et al.*, 1993; Bates *et al.*, 1993; Smith and Zilfou, 1995; Sha *et al.*, 1996). A subset of staurosporine derivatives have also been shown to inhibit binding of [³H] vinblastine. The PKC inhibitors Ro31-8220 and UCN-01 are transported by Pgp as suggested by the resistance of MCF-7/ADR cells to cytostasis induced by these compounds (Budworth *et al.*, 1996) though in KB MDR cells we found that [¹⁴C]-Ro31-8220 had a significantly higher affinity for Pgp than the parent staurosporine as judged by inhibition of [³H] azidopine binding (Drew, 1996). We also found that Ro31-8220 was transported by Pgp (Drew, 1996). Such inhibitors may thus alter drug accumulation independently of, or in addition to, their effects on PKC and the phosphorylation state of Pgp, by interacting directly with Pgp. This is suggested by Wakusawa *et al.* (1993) who found there was no correlation between the ability of staurosporine derivatives to inhibit protein kinase activities *in vitro* and the effect on vinblastine accumulation in P388/ADR cells.

Furthermore, comparison of staurosporine and the derivatives UCN-01, GF109203X and CGP 41251 which have similar PKC-inhibition properties, differed widely in both their ability to reverse Pgp-

mediated drug resistance and their susceptibility to transport by Pgp suggesting that inhibition of PKC is not a major mechanism by which these compounds modulate MDR at least in MCF-7/ADR cells (Budworth *et al.*, 1996). We found much the same with a series of Roche staurosporine derivatives: there was no clear correlation between PKC inhibitory activities *in vitro* and the effect on daunomycin accumulation and efflux in KB-C1 cells (Drew, 1996). The inactive precursor of the PKC inhibitor calphostin C increased daunorubicin accumulation, decreased efflux of daunorubicin and partially reversed daunorubicin resistance in P388/ADR cells (Gupta *et al.*, 1994b). Early studies with verapamil, also a PKC inhibitor, must be interpreted cautiously since this is a potent chemosensitising agent and binds to Pgp directly, probably producing its effects on drug accumulation by competition (Hamada *et al.*, 1987). Most recently, however, Beltran *et al.* (1997) have reported that CGP41251 does not block [³H] azidopine photoaffinity labelling of Pgp at a concentration of 125 nM at which it induced drug uptake and decreased Pgp phosphorylation supporting a role for phosphorylation in maintaining the MDR phenotype.

Results gained using some protein kinase inhibitors described above present a confusing picture of the involvement of PKC in regulating MDR via Pgp almost certainly because of their ability to bind to, and be transported by Pgp. Further, protein kinase inhibitors such as staurosporine can also influence MDR1 gene expression and may thus alter drug accumulation independently of effects on Pgp phosphorylation (see below). However, use of safinol and sphingosine stereoisomers which inhibit at the regulatory site of PKC and are not substrates for Pgp suggest that enhanced drug accumulation and sensitivity in MCF-7/DOXR cells is associated with the inhibition of PKC-mediated phosphorylation of Pgp rather than any competitive interference with Pgp drug binding or effect on Pgp expression (Sachs *et al.*, 1995b). Also an N-myristoylated PKC- α pseudosubstrate peptide partially reversed drug resistance, increased cellular accumulation and decreased Pgp phosphorylation in MCF-7/DOXR cells but did not bind to Pgp or alter Pgp expression (Gupta *et al.*, 1996). At least in MCF-7 MDR cells such findings suggest that phosphorylation of Pgp by PKC may regulate Pgp activity as initially thought though this may be by influencing the ATPase and drug binding activities of Pgp (O'Brian *et al.*, 1994). This may be true for other MDR cell lines as the effects of CGP 41251 on doxorubicin sensitivity

and accumulation in drug sensitive and MDR CT-26 and UV2237M cells were thought to be independent of binding to Pgp directly (Killion *et al.*, 1995).

Phorbol esters and MDR

Phorbol ester activators of conventional and novel PKC subspecies confer or enhance resistance to MDR-associated drugs in drug-sensitive and MDR cells respectively. Phorbol esters activate PKC subspecies by acting as DAG analogues but results obtained by this approach can be difficult to interpret since phorbol esters are not readily metabolised and initiate downregulation of susceptible PKC subspecies within a short time of application (see above). Further, phorbol esters can bind to Pgp in drug-resistant cells leading to difficulties in interpreting results on drug transport.

Short-term phorbol ester exposure of drug sensitive and MDR MOLT3, MCF-7 and UV-2237M cells and prolonged treatment of drug sensitive KM1214A cells induced drug resistance under conditions of PKC activation and not downregulation (Schwartz *et al.*, 1991; Dong *et al.*, 1991; Fine *et al.*, 1988; Ahn *et al.*, 1996). In addition, short-term treatment of KB-3-1 cells with TPA induced transient protection to VP-16 (Ferguson and Cheng, 1987). Short term incubation with TPA also protected P388 cells against the cytotoxic effects of daunorubicin for 60 min though when exposure to the drug was increased to 24 hr, TPA treatment did not alter daunorubicin toxicity (Kessel, 1988). Treatment of tumour cells with phorbol esters typically increases IC₅₀ values of cytotoxic drugs affected by MDR by 1.3 to 4.0 fold. In contrast, however, activation of PKC in S180A10 cells by short term TPA treatment enhanced cytotoxicity to doxorubicin while downregulation of PKC by long-term TPA treatment increased protection to doxorubicin-induced cytotoxicity (Posada *et al.*, 1989b).

Phorbol ester-mediated protection of drug-sensitive and MDR cells is generally associated with a reduction in the intracellular accumulation of drugs (Ido *et al.*, 1986; Ferguson and Cheng, 1987; Fine *et al.*, 1988; Chambers *et al.*, 1990a; O'Brian *et al.*, 1994; Dong *et al.*, 1991). The effect is usually seen after exposure of around one hour and is abolished after long incubations. An exceptional observation that the TPA effect was maintained over 24 hr in KB-V1 cells may be attributed to the unusual resistance to downregulation of PKC in these cells (Chambers *et al.*, 1990b; Chambers, 1995). Several studies have shown that equivalent concentrations of inactive phorbol esters did not

affect drug accumulation, supporting the view that phorbol ester-induced reduction of drug accumulation is a direct consequence of PKC activation. However, the PKC inhibitor H-7 did not inhibit the reduction in drug accumulation mediated by TPA in MCF-7/Dox cells and the TPA-induced protection to drugs observed in KB-3-1 cells could not be reversed by the PKC inhibitor tamoxifen or mimicked by the synthetic DAG, OAG (Choi *et al.*, 1993; Ahn *et al.*, 1996; Ferguson and Cheng, 1987) making it unclear whether PKC was involved in the phorbol ester-mediated effect. These inconsistencies may reflect the uncertainty of the precise effects which phorbol esters may exert on cells and, in most MDR cells, their ability to bind to Pgp.

In a detailed study of the effect of PKC activators and inhibitors on the reversal of MDR, Smith and Zilfou (1995) noted that phorbol esters produced contrary effects to the trends described above, namely, MDR was reversed and drug accumulation increased by phorbol ester treatment of MCF-7/ADR cells, in association with enhanced Pgp phosphorylation. Such effects were only observed when μM concentrations of TPA were used and staurosporine did not block the TPA-mediated effects observed suggesting that the findings were independent of PKC. Smith and Zilfou (1995) found that both active and inactive isomers of TPA and PDBu appeared to bind to the drug binding site of Pgp in MCF-7/ADR cells but were not transported by Pgp. Epand and Stafford (1993) have drawn similar conclusions from studies with Chinese hamster CHRC5 cells where both active and inactive phorbol esters increased rhodamine accumulation at mM concentrations.

Clear evidence that phorbol esters activate PKC to enhance drug efflux by phosphorylation of Pgp has yet to be obtained. TPA treatment of MCF-7/ADR cells enhanced drug efflux activity with enhanced Pgp phosphorylation which could be inhibited by staurosporine (Aftab *et al.*, 1994), a finding which did not require new synthesis of Pgp. This is suggestive of a role for PKC-mediated enhancement of Pgp activity through phosphorylation. However, a more recent report suggests that altered Pgp phosphorylation by PKC does not affect drug transport (Scala *et al.*, 1995a). Prolonged treatment of MCF-7TH cells with the PKC activator/inhibitor Bryostatins 1, decreased phosphorylation of Pgp and caused downregulation of PKC- α but had no effect on Pgp function as measured by vinblastine and rhodamine 123 accumulation. It was established some time ago (Fine *et al.*, 1988) that

the MDR phenotype in these MCF-7TH MDR cells is partly associated with a 20 kDa protein which is phosphorylated by PKC.

PKC and MDR1 gene expression

PKC may also have a role in the transcriptional activation of the MDR1 gene (Grunicke *et al.*, 1994) and thus in longer term experiments with phorbol esters effects due to increased synthesis of Pgp protein must be considered. Exposure of KB-V1 cells to staurosporine diminished Pgp protein and decreased MDR1 mRNA (Sampson *et al.*, 1993b). Similarly the PKC inhibitor dextrageldipine hydrochloride decreased Pgp levels within 30 min of treating GM86E cells though this effect was not maintained (Patterson *et al.*, 1996). CGP41251 treatment of CCRF-VCR1000 cells for 12, 24 and 48 hr did not alter expression of the MDR1 gene (Utz *et al.*, 1994; Grunicke *et al.*, 1994). TPA and DAG activators of PKC increased MDR1 mRNA and Pgp protein levels in normal peripheral blood cells and cell lines derived from leukaemias and solid tumours, an effect blocked by staurosporine suggesting regulation via a PKC-mediated pathway (Chaudhary and Roninson, 1992). PKC subspecies α , β and/or ζ have been implicated in mediating the TPA-induced increase in Pgp expression which occurs in MCF-7/Dox cells as this effect was not blocked by H-7 which inhibits translocation of PKCs δ and ϵ but not α and β (Ahn *et al.*, 1996). A role for PKC in modulating MDR1 gene expression has further been suggested following the observation of sequential expression of PKC and Pgp in AH66 MDR cells and AH66 cells exposed to doxorubicin (Ohkawa *et al.*, 1994a, 1994b). A role for the trans-acting factor EGR1 in mediating TPA activation of MDR1 gene expression in K562 cells has been reported (McCoy *et al.*, 1995). Germann *et al.* (1995) observed that the activation of Pgp expression may underlie the observations that phorbol ester treatment of some drug-sensitive cell lines which do not express Pgp prior to exposure can lead to acquisition of an MDR-like phenotype. This is not always the case, however. For example, we treated drug-sensitive KB-3-1 cells with 500 nM TPA for up to 24 hr: no expression of Pgp by western blotting was detected, even on overexposed blots (Drew *et al.*, 1996) but drug influx was reduced. Additionally, 500 nM TPA treatment did not increase Pgp expression in drug-resistant KB-C1 cells.

In relation to mechanisms by which Pgp may become overexpressed in MDR, Osborn and Chambers

(1996) have recently shown that the stress activated kinase JNK may play a part in the response to drugs used in chemotherapy. Treatment of drug-sensitive KB-3 cells with doxorubicin activated JNK up to 40 fold: vinblastine and etoposide also activated JNK but to a lesser extent. c-Jun phosphorylation was also increased indicating activation. The drug resistant KB-A1 and V1 MDR lines showed increased JNK activity. Interestingly, under optimal conditions for JNK activation all the drugs used induced MDR1 mRNA expression in the drug-sensitive KB-3 cells linking exposure to drugs to activation of Pgp expression.

PKC induces drug resistance independent of Pgp

Where studied, it seems that phorbol ester treatment or PKC overexpression can induce a MDR phenotype in drug sensitive cells without either inducing or altering Pgp expression. Overexpression of PKC- β_1 in drug-sensitive fibroblasts conferred a multidrug resistance phenotype including resistance to doxorubicin, vinblastine, actinomycin D and vincristine but not 5-fluorouracil on rat fibroblasts without either inducing or altering levels of Pgp expression (Fan *et al.*, 1992). In this work a decreased rate of drug uptake was found to account for the reduction in drug accumulation. PKC may therefore modulate other multidrug resistance mechanisms that function independently of Pgp. Phorbol ester stimulation reduced drug uptake into drug-sensitive MCF-7 WT, P388 and KM12L4a cells without affecting efflux rates (Fine *et al.*, 1988; Dong *et al.*, 1991). In MCF-7WT cells activation of PKC increased phosphorylation of a 20 kDa membrane protein. A synthetic diacylglycerol, OAG, reduced drug uptake in KM12L4a cells without altering drug efflux (Dong *et al.*, 1991). It is therefore possible that activation of PKC by TPA influences MDR through Pgp-independent mechanisms which may relate, for example, to regulation of other membrane transport proteins and/or to membrane lipid changes. This mechanism may also modulate drug accumulation in MCF-7/ADR cells where reduced drug influx in addition to enhanced efflux was observed on TPA treatment (Hait *et al.*, 1993). Treatment of drug-sensitive P388 cells with deoxyphorbol derivatives has also been found to induce resistance to daunorubicin and was associated with translocation of certain PKC subspecies to the membrane (Gollapudi *et al.*, 1994a, b).

We have noted that TPA treatment of drug-sensitive KB-3-1 and drug resistant KB-C1 cells re-

duced daunomycin accumulation but did not influence drug efflux or Pgp expression: the TPA effect was reversed by Ro31-8220 while inactive 4 α -TPA was without influence (Drew *et al.*, 1996). We have suggested that PKC can regulate drug influx by mechanisms which are independent of Pgp and that this may represent a mechanism of drug resistance which can operate independently of, or in addition to, Pgp-mediated drug efflux.

How is PKC activated in MDR?

If PKC plays a key role in Pgp-independent or Pgp-dependent drug resistance mechanisms, it will need to be in a constantly activated state to continually phosphorylate components of drug-efflux mechanisms. To achieve this activated state current thinking (see above) suggests that PKC must, i) become autophosphorylated, having first been phosphorylated at threonine-497 (for PKC- α) by an undefined PKC kinase and ii) for conventional and novel subspecies at least, interact with DAG at sites of PKC-substrate interactions, which may also involve special binding/receptor proteins. It is of interest that Ahmad *et al.* (1994) noted that the PKC- α , overexpressed in membrane vesicles from a baculovirus expression system with Pgp, was itself phosphorylated suggesting that initial activation at least had occurred. However, little is known of how DAG is supplied in drug-resistant cells to sustain PKC activation. DAG is rapidly metabolised and thus mechanisms to ensure constant formation of this key lipid second messenger must be operating.

In this context the reports that many cells with the MDR phenotype overexpress receptors for epidermal growth factor (Meyers *et al.*, 1986, 1988; Dickstein *et al.*, 1993; Vickers *et al.*, 1988) is perhaps relevant. This may arise as a result of drug exposure since doxorubicin has been shown to upregulate receptors for epidermal growth factor (Zuckier and Tritton, 1983). Yang *et al.* (1997) showed recently that stimulation of MDR cells with EGF results in stimulation of PLC and the hydrolysis of PtdIns-4,5-P₂ to generate DAG and Ins 1,4,5-P₃: at the same time a transient increase in Pgp phosphorylation and then a dephosphorylation was observed as well as a decreased drug accumulation. Meyers *et al.* (1993) found that treatment of MDR cells with EGF can also activate protein phosphatases-1 and -2A which bring about Pgp dephosphorylation but at certain time points Pgp phosphorylation was also observed. The DAG produced in the EGF response would provide for short-term PKC

stimulation in normal cells but in MDR cells overexpression of the EGFR may provide a mechanism for a more sustained DAG formation for PKC activation.

PKC activated by DAG produced via activation of the EGF receptor would then be available to activate phospholipase D (Kiss, 1996; Exton, 1997). This is relevant since PLD activity is enhanced 4–6 fold in drug-resistant MCF-7/Adr cells (Welsh *et al.*, 1994; Kiss *et al.*, 1994). Welsh and colleagues also noted that phorbol ester treatment of the MDR cells resulted in an increased mass of cellular DAG compared with wild-type cells suggesting that pathways to convert the product of PLD activity, phosphatidate, to DAG by phosphatidate phosphohydrolase were operative. Without phorbol ester stimulation PLD activities in drug sensitive and MDR lines were the same suggesting that the presence of Pgp might promote or accelerate coupling of the phorbol ester stimulus to PLD activation in the MDR cells. Of further interest is the fact that the MDR MCF-7 cells contain a phorbol ester-sensitive phosphatidylethanolamine-specific PLD activity (Kiss *et al.*, 1994): both wild-type MCF-7 and MCF-7/MDR lines contained a low level of phosphatidylcholine-specific PLD activity which was not phorbol ester sensitive. Kiss *et al.* (1994) speculate that PtdEtn hydrolysis may have a special function in drug-resistant MCF-7 cells contributing to the MDR phenotype perhaps via the fusogenic properties of phosphatidate or alterations in plasma membrane structure modulating Pgp transporter activity. Further, PtdEtn can activate the ATPase activity of Pgp (Doige *et al.*, 1993; Sharom *et al.*, 1995). Phospholipase C in MCF-7/Adr cells can also be activated by cell stress, notably heat shock, to increase DAG levels, PKC activation and Pgp phosphorylation (Yang *et al.*, 1995). Since exposure to drugs activates the stress-activated JNK pathway (Osborn and Chambers, 1996) resulting in induction of MDR1 mRNA it is now important to discover if such drug-induced stress activates phospholipase C leading to DAG formation to activate PKC.

Pgp clearly has a special interaction with PtdEtn in plasma membranes (Sharom *et al.*, 1995) and PtdEtn may be vital in regulating Pgp activity since inostamycin is postulated by Kawada and Umezawa (1995) to inhibit Pgp function by binding irreversibly to PtdEtn. Activation of a PtdEtn-specific PLD by PKC leading to PtdEtn hydrolysis to phosphatidate could significantly influence Pgp function by altering the lipid microenvironment around the transporter in the membrane. Pgp itself is now clearly defined as a

broad specificity lipid translocase (van-Helvoort *et al.*, 1996).

PKC, apoptosis and drug resistance

PKC is thought to oppose pathways by which cells undertake apoptosis or programmed cell death (Grant and Jarvis, 1996) and such observations may be of relevance in newer therapeutic strategies to overcome the problems of MDR. Activation of PKC can often prevent cells from entering programmed cell death while many PKC inhibitors are potent inducers of apoptosis. Recently it has become clear that PKC and ceramide have opposing actions in regulating apoptosis (Hannun and Obeid, 1995) and changes in ceramide glycosylation have been found in MDR cells (Lavie *et al.*, 1996, 1997). We have found that the preferential killing of a range of MDR KB cells by 2-deoxyglucose-induced apoptosis correlates strongly with the PKC levels of these cells (Drew *et al.*, 1994; Bell, S., unpublished). This may reflect the wider cellular consequences of elevated PKC levels in MDR cells.

PKC and other aspects of drug resistance

Other drug transporting molecules such as the multidrug resistance associated protein MRP (Chapter 3) and the lung resistance-related protein, LRP (Chapter 5) are implicated in the broader area of drug resistance, besides Pgp in the MDR phenotype. MRP is reported to be highly phosphorylated at serine residues and this phosphorylation was reduced by the kinase inhibitor H-7 and by the PKC inhibitor chelerythrine (Ma *et al.*, 1995). These inhibitors also increased drug accumulation and decreased drug efflux suggesting that certain phosphate residues on MRP may modulate drug accumulation in HL-60-ADR cells. This was also suggested by Gekeler *et al.* (1995) where the more specific PKC inhibitor GF 109203X reversed vincristine resistance and modulated rhodamine 123 efflux in HL-60/ADR cells without influencing MRP gene expression. There is no evidence yet whether PKC inhibitors interact directly with MRP. Changes in the expression of PKC subspecies in MRP-expressing LoVo cell lines has just been reported (Dolfini *et al.*, 1997). There is yet little evidence whether kinases are involved in the action of the lung resistance related protein (LRP) which now turns out to be the major vault protein as described in Chapter 5.

Glutathione transferase and topoisomerase II are both potential substrates for PKC which have been implicated in drug resistance. Topoisomerase II is a

substrate for PKC *in vitro* and is phosphorylated *in vivo* (Heck *et al.*, 1989). However, TPA inhibited rather than stimulated etoposide-mediated DNA cleavage in HL-60 cells (Zwelling *et al.*, 1990). Etoposide resistance was also associated with decreased PKC activity in P388 cells but no link between PKC and topoisomerase activity was reported (Ido *et al.*, 1987). Furthermore, although mutant forms of topoisomerase II from etoposide-resistant KB cells had higher activities and also contained increased levels of serine phosphorylation in intact cells compared to the wild-type enzyme, phorbol esters did increase phosphorylation (Takano *et al.*, 1991). Clearly such findings need further examination.

PKA and MDR

PKA

PKA is a ser/thr kinase in the cytosol. Here it is activated by cAMP formed by adenylyl cyclase via a G-protein-coupled signalling pathway triggered by activation of 7-span receptors, such as the β -adrenergic receptor, in the plasma membrane. PKA is composed of a regulatory (R) subunit and a catalytic (C) subunit and the overall tetrameric structure of PKA made up of a dimer of two R units, complexed with two C units. This complex is inactive until two cAMP molecules bind to each R unit. This causes the dissociation of the complex to leave two catalytically-active C subunits which phosphorylate a range of substrates regulating metabolism, ion channel function, cell proliferation and gene induction. Active C subunits catalyse some of these events in the nucleus. PKA is present in mammalian cells as two distinct isoforms, type I and type II, which have different R subunits, R-I and R-II. Type I PKA is linked to the regulation of cell growth processes while type II PKA regulates cell maturation and differentiation. Type I PKA has a higher affinity for cAMP than the type II form and turnover rates also differ: such functional differences are probably related to the transient or sustained responses exhibited by PKA to hormones. The ratio of type I to type II PKA can change during cell development and in neoplasia indicating that the two PKA isoforms may have separate roles. Four isoforms of the R subunit of PKA (R-I α , R-I β , R-II α and R-II β) and three C subunit isoforms (C α , C β , C γ) have been identified. These differ in type 1 and type 2 PKA in different tissues. Type 1 PKA in the cytosol as well as type II PKA associated

with Golgi and membranes has C α and C β isoforms. Rohlf and Glazer (1995) have briefly reviewed the structure of PKA.

PKA phosphorylation of Pgp

Phosphorylation of mouse Pgp is stimulated by cAMP and the C-subunit of PKA can phosphorylate Pgp (Mellado and Horwitz, 1987). More recently, Chambers *et al.* (1994) have shown that purified PKA phosphorylates serine residues in the linker region of human Pgp. Using synthetic peptides corresponding to residues 656-689 serines 667, 671 and 683 were phosphorylated by PKA. Of these sites serines 667 and 671 were also phosphorylated by PKC (see above) but ser-683 was PKA-specific. The synthetic peptide acted as a competitive substrate for Pgp phosphorylation when incubated with membrane vesicles formed from the drug-resistant KB-V1 cell line; endogenous kinases associated with the KB-V1 membranes phosphorylated serines 661, 667 and 671 but only low phosphorylation of the PKA-specific serine 683 site was detected. Pgp in KB-V1 cell membranes was not phosphorylated at the PKA-specific site at all but was phosphorylated at the PKC sites (Chambers *et al.*, 1994). The endogenous kinases associated with the KB-V1 cell membranes used in such studies were not characterised in the work but probably included the 55-60 kDa V-1 kinase and the 170 kDa kinase already identified in drug-resistant KB-V1 cells (see below). Some PKC activity associated with Pgp (see above) may also have been present but it is not clear if any residual PKA activity remained in the vesicle preparations.

Alteration of PKA expression in multidrug resistant cells

Unlike PKC, there have been few reports on whether PKA activity and protein expression is routinely altered in the MDR phenotype. In one study, the regulatory subunit of PKA, R-1 α , was increased five to ten-fold in Pgp-expressing HL-60/VCR, KB-V1 and MCF-7 Vbl cells as well as Pgp-unassociated drug-resistant HL-60/ADR cells compared with drug-sensitive parental lines (Budillon *et al.*, 1992). We have measured PKA activity using the peptide substrate kemptide in a series of increasingly vincristine-resistant CHO cell lines (Fenton, 1996) as well as the E29 drug-sensitive parental line. PKA activity was significantly increased in the less resistant VRT5 and VRT15 lines which did not overexpress PKC but

was not significantly enhanced in the most drug resistant VRT25 line which showed a 3–4 fold increase in PKC overexpression. This finding suggests that a possible reciprocal relationship between PKA and PKC may operate in drug-resistant cells, though to establish this fully many other cell lines need examination. Immunoblotting indicated that type I PKA was expressed but there was no increase in protein expression between drug-sensitive and drug-resistant lines suggesting that the observed increase in measurable PKA activity with kemptide might be due to type II PKA expression which we were unable to monitor by immunoblotting at the time.

The PKA inhibitor H-87 has been found to potentiate the cytotoxic effects of some MDR drugs including vinblastine and doxorubicin but not etoposide and some non-MDR drugs in AH66 and P388 MDR cells (Nakamura *et al.*, 1993, 1996): reversal of drug resistance was associated with increased drug accumulation and decreased efflux. However, such studies have the same problem as many inhibitors to examine the role of PKC in MDR using. Namely, the inhibitor interacts directly with Pgp and its observed effect is probably mediated by competition with drugs for binding to Pgp (Nakamura *et al.*, 1996) as well as by some inhibition of PKA. Primary breast carcinomas are reported to have altered ratios of type I to type II PKA while high levels of cAMP-binding proteins in breast tumours were correlated with a poor prognosis (in Rohlff and Glazer, 1995).

PKA regulation of Pgp expression

The relationship between PKA and MDR1 gene transcription is currently being explored in some detail and a role for PKA in signalling pathways regulating MDR1 gene expression is clearly emerging as summarised by Rohlff and Glazer, (1995). Abraham *et al.* (1990) showed that transfection of CHO cells with a mouse mutant regulatory subunit (R-1) increased drug sensitivity and decreased Pgp expression at both mRNA and protein levels: it was suggested that normal PKA activity is required to sustain basal levels of Pgp. H-87, a PKA inhibitor, reduced MDR1 gene transcription (Kim *et al.*, 1993) while 8-Cl-cAMP, a site-selective analogue of cAMP, caused growth inhibition in a range of Pgp associated and non-Pgp associated MDR cell lines (Yokozaki *et al.*, 1993). The 8-Cl-cAMP treatment led to downregulation of R-I α subunit protein expression, reduced PKA activity and suppression of the promoter activity of the

MDR1 gene transfected into MCF-7 cells. This has been confirmed in a more recent study showing that 8-Cl-cAMP decreased MDR1 mRNA expression, reduced synthesis of Pgp and an increased vinblastine accumulation in MCF-7TH cells (Scala *et al.*, 1995b). Here a change in the ratio between the R-I and R-II regulatory subunits of PKA was observed, though PKA catalytic activity was unchanged over 48 hr of treating cells with 8-Cl-cAMP. This supports the view that MDR1 gene expression is regulated in part by changes in PKA isozyme levels. Expression of active C subunit of PKA in Y1 cells expressing defective regulatory subunits restored MDR1 RNA levels indicating a cause and effect relationship between the kinase mutations and MDR1 gene expression (Chin *et al.*, 1992). Such studies implicate PKA in the MDR phenotype at the level of regulating MDR1 transcription rather than by phosphorylation effects on Pgp (Rohlff and Glazer, 1995). Full details of the transcriptional regulation of *mdr* genes are described in Chapter 7 and it seems that PKA exerts effects on MDR1 gene transcription through several factors, for example, through the PKA-dependent transcription factor CREB, through AP-1 and through a cAMP-responsive sequence (CRS) which is similar to the Sp1 element (Rohlff and Glazer, 1995). Several Sp1 sites have been identified upstream of the MDR1 gene and a recent report (Rohlff *et al.*, 1997) shows that levels and DNA binding activity of Sp1 are increased in HL-60/AR drug-resistant cells and that in such cells the trans-activating and DNA binding properties of Sp1 are stimulated by PKA. Sp1 is thus a cAMP-responsive transcription factor and such studies again emphasise the regulatory role of PKA in MDR1 transcription. Such studies indicate the potential of PKA as a target for reversal of multidrug resistance using cAMP analogues and 8-Cl-cAMP is already in clinical trial for this purpose (Rohlff and Glazer, 1995).

Other kinases and drug resistance

A few other kinases have been implicated in MDR though studies are few and it is not at all clear what role such kinases play in the MDR phenotype, if any. PK-I, a kinase detected in both drug-sensitive and drug-resistant HL-60 cells which can phosphorylate Pgp in membranes of the drug-resistant cells (Staats *et al.*, 1990) may be similar to a 27 kDa protein kinase of platelets (Elias and Davis, 1985). PK-1 phosphorylation of endogenous membrane proteins is

enhanced by phospholipid and by Mn^{2+} ions but is calcium-independent. PK-1 can phosphorylate synthetic peptides corresponding to sequences from Pgp though these were not in the linker region and phosphorylation is at both serine and threonine residues.

Two membrane-bound kinases in drug resistant KB-V1 cells have been reported. A 170 kDa serine/threonine kinase expressed in drug-resistant KB-V1 cells, but not in the drug-sensitive KB-3-1 line, was also elevated in MCF-7/ADRR and B16/ADRR cells but not drug-sensitive or MDR P388 cells. Its pattern of expression correlates with levels of multidrug resistance but it is not clear if Pgp is a substrate of this kinase (Sampson *et al.*, 1993) which is calcium-, phosphatidylserine- and cAMP-independent but inhibited by staurosporine, K525a and KT5720 (Sampson *et al.*, 1993). A 55–60 kDa V-1 kinase present in both drug-sensitive KB-3-1 and drug-resistant KB-V1 cells can phosphorylate Pgp *in vitro* but not a mutant Pgp lacking serine residues 661, 667, 671, 675 and 683 in the linker region (Germann *et al.*, 1996): histone, or PKC and PKA peptide substrates were also not phosphorylated (Germann *et al.*, 1995).

GTP can replace ATP as an energy source to support drug transport by Pgp in membrane vesicles from drug-resistant KB-V1 cells and a GTP-dependent kinase may contribute to the overall phosphorylation of Pgp *in vitro* (Lelong *et al.*, 1994).

Tyrosine kinase inhibitors, such as genistein, have been found to enhance the cytotoxicity of drugs in K562/TPA cells in which MDR was thought to be independent of Pgp, MRP and topoisomerase II (Takeda *et al.*, 1994). Genistein may also reverse drug-resistance in non-Pgp expressing human colon carcinoma MDR S1-M1 cells by increasing drug accumulation (Rabindran *et al.*, 1995). Further, the transient protection of KB-3-1 cells to antitumour agents by TPA was thought not to be due to PKC but a possible role for tyrosine kinases was considered since TPA can directly stimulate tyrosine kinase activity in membrane preparations (Ferguson and Cheng, 1987). Tyrosine kinases in MDR may regulate novel drug resistance pathways as genistein did not modulate resistance in Pgp expressing K562/ADM or MRP-expressing HL-60/ADR cell lines.

Finally, the ras protein may play a role in MDR through activation of the serine/threonine kinase c-raf-1 which activates the MAP kinase signalling pathway to influence transcriptional expression of cellular genes via the AP1 binding site. The promoter region of the MDR1 gene contains an AP1 binding site

and Ras stimulates transcriptional activation of the MDR1 gene to induce resistance to a wide variety of anticancer drugs (Stromskaya *et al.*, 1995). c-Jun overexpression has been linked to MDR1 overexpression in U937 cells (Slapak *et al.*, 1993) while c-Fos and c-Jun expression was associated with doxorubicin resistance in non-small cell lung carcinomas of untreated patients (Volm and Pommerenke, 1995). PKC subspecies α , ϵ and ζ can phosphorylate Raf to activate the MAP kinase pathway. More recently, Kim *et al.* (1996) have proposed that Raf-dependent, as well as PKA-dependent pathways control transcription of the MDR1 gene via a mechanism involving modulation of heat-shock factor activity. Chin *et al.* (1990) noted some time ago that heat-shock leads to an increase in MDR1 gene expression and identified several heat shock consensus elements in the promoter region of the human MDR1 gene. Heat shock protein 90 beta is expressed constitutively in a doxorubicin-resistant LoVo DX(R) line but not in the sensitive parental line: hsp90 beta can be induced in the drug sensitive line by exposure to stress such as drugs and heat shock (Bertram *et al.*, 1996). Interestingly, in the drug resistant cells hsp90 is tightly associated with Pgp as shown by their co-immunoprecipitation. Such association may form a mechanism for modulation of Pgp function which needs more detailed examination.

Conclusions

The involvement of PKC in the MDR phenotype has received much attention but, in spite of all the work, few really conclusive findings have emerged and results are varied and often confusing. This may be because the role of PKC in MDR varies from cell type to cell type and is also dependent on the drug involved in selection. It is clear however that problems generated by the binding of inhibitors and activators to Pgp are not well understood and make interpretation of results extremely difficult. The same is true for studies on PKA where inhibitors are used. The mechanism by which PKC regulates MDR1 gene expression at both transcriptional and translational levels needs much fuller investigation as does the question of whether the elevated protein expression of specific PKC subspecies in drug-resistant cells arises from increased synthesis, alterations in mRNA turnover or decreased PKC proteolysis. Evidence is accumulating to indicate that PKC can regulate a Pgp-independent form of drug resistance. This needs to be firmly estab-

lished, the precise mechanism(s) elucidated and PKC subspecies involvement defined since it may provide a further rationale for the use of PKC inhibitors in the clinic as anticancer agents (Basu, 1993). PKA receives relatively little attention compared with PKC, yet its role in regulating MDR1 gene transcription seems established making it a potentially good therapeutic target. Clearly PKA involvement in MDR needs much more attention. This is also true of the several minor kinases which have been identified in MDR cells and which need better characterisation. Effects from some of these minor kinases may further confuse results obtained with phorbol esters and kinase inhibitors.

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