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Effects of Phosphorylation of P-glycoprotein on Multidrug Resistance

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Cells expressing elevated levels of the membrane phosphoprotein P-glycoprotein exhibit a multidrug resistance phenotype. Studies involving protein kinase activators and inhibitors have implied that covalent modification of P-glycoprotein by phosphorylation may modulate its biological activity as a multidrug transporter. Most of these reagents, however, have additional mechanisms of action and may alter drug accumulation within multidrug resistant cells independent of, or in addition to, their effects on the state of phosphorylation of P-glycoprotein. The protein kinase(s) responsible for P-glycoprotein phosphorylation has(ve) not been unambiguously identified, although several possible candidates have been suggested. Recent biochemical analyses demonstrate that the major sites of phosphorylation are clustered within the linker region that connects the two homologous halves of P-glycoprotein. Mutational analyses have been initiated to confirm this finding. Preliminary data obtained from phosphorylation- and dephosphorylation-defective mutants suggest that phosphorylation of P-glycoprotein is not essential to confer multidrug resistance.

KEY WORDS: Multidrug resistance; P-glycoprotein; multidrug transporter; protein kinase C; cAMP-dependent protein kinase; phosphorylation sites; linker region.

INTRODUCTION

Multidrug resistance (mdr) is a major obstacle to effective cancer chemotherapy. In many cultured cell lines mdr has been associated with overexpression of P-glycoprotein (Nielsen and Skovsgaard, 1992). Human P-glycoprotein is encoded by the *MDR*1 gene and represents an integral plasma membrane transport protein that recognizes chemically diverse natural product cytotoxic agents as substrates for ATP-dependent efflux (Gottesman and Pastan, 1993; Germann, 1993; Childs and Ling, 1994). Thus, multidrug-resistant cells characteristically exhibit reduced levels of accumulated drug when compared with parental cells. Gene transfer experiments involving *mdr* cDNAs have confirmed that expression of P-glycoprotein is sufficient to render drug-sensitive cells multidrug resistant (Gros *et al.*, 1986a; Ueda *et al.*, 1987; Guild *et al.*, 1988; Pastan *et al.*, 1988). P-glycoproteins (Pgp) encoded by highly homologous *mdr* or *pgp* genes have also been found in rodents (Gros *et al.*, 1986a,b,c; Endicott *et al.*, 1987; Hsu *et al.*, 1989; Devault and Gros, 1990; Silverman *et al.*, 1991; Deuchars *et al.*, 1992). The *mdr* genes comprise a subfamily of a large superfamily known as *A*TP *b*inding *c*assette (ABC) transporters, or traffic ATPases (Hyde *et al.*, 1990; Mimura *et al.*, 1991). To date, over 50 ABC transporters from prokaryotic or

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eukaryotic origin have been identified (for reviews see Ames *et al.*, 1992; Higgins, 1992). Depending on their physiologic transport function, they are located at the cell surface, or within intracellular membranes (e.g., peroxisomes, endoplasmic reticulum). ABC transporters typically consist of four core domains, two hydrophobic transmembrane domains with multiple membrane-spanning segments, and two hydrophilic domains located on the cytplasmic face of the membrane. These cytosolic domains are homologous with each other (overall ~30% identical) and contain nucleotide binding motifs (Walker *et al.*, 1982). The P-glycoprotein nucleotide binding folds have been shown to bind ATP, a prerequisite for coupling of ATP hydrolysis to the transport process (Gottesman and Pastan, 1993; Germann, 1993).

Based on the primary structure deduced from the cDNA sequence, the human *MDR*1 gene product is composed of two halves that exhibit striking sequence similarities (Chen *et al.*, 1986). As shown in Fig. 1, both halves consist of an N-terminal membrane spanning domain that is followed by a C-terminal cytosolic nucleotide binding fold. Soon after P-glycoprotein was first discovered (Juliano and Ling, 1976), it was described as a phosphoglycoprotein (Carlsen *et al.*, 1977). Several cell-biological studies have indicated that glycosylation of P-glycoprotein is not pivotal for its drug transport function (Beck and Cirtain, 1982; Ling *et al.*, 1983; Ichikawa *et al.*, 1991). Recent data from mutational analyses of the major glycosylation sites within the first extracytoplasmic loop of P-glycoprotein suggested that glycosylation may contribute to targeting of the protein to the cell surface, and stabilizing the membrane-associated polypeptide chain against proteolytic digestion (Schinkel *et al.*, 1993). In contrast, the role of the post-translational modification of P-glycoprotein by phosphorylation remains unclear to date, and has been the subject of some controversy, as will be described in this minireview.

MODULATION OF MULTIDRUG RESISTANCE BY PROTEIN KINASE ACTIVATORS AND INHIBITORS

Protein phosphorylation plays a crucial role in the execution and regulation of many cellular functions. As already noted, P-glycoprotein was shown to be phosphorylated (Carlsen *et al.*, 1997) soon after its discovery in Chinese hamster ovary cells selected for resistance to colchicine (Juliano and Ling, 1976). Subsequently, this covalent modification has been established as a general characteristic of native P-glycoprotein produced in intrinsically drug-resistant or drug-selected cell lines of human and rodent origin

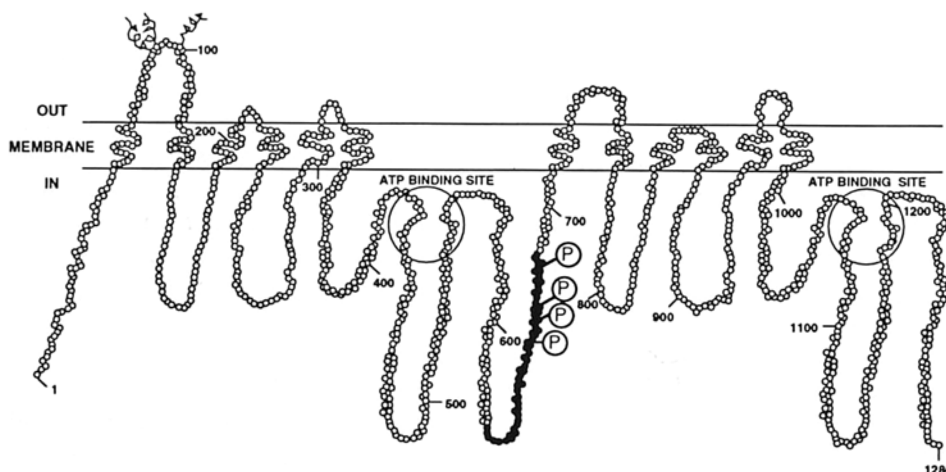

Fig. 1. Two-dimensional model of human P-glycoprotein inserted into the plasma membrane with putative phosphorylation sites. The model is based on hydropathy analysis of the P-glycoprotein amino acid sequence deduced from the *MDR*1 cDNA sequence (Chen *et al.*, 1986). The ATP binding sites are circled and the putative N-linked glycosylation sites are represented by wiggly lines. Darkened circles represent the P-glycoprotein linker region encoded by exon 16 (amino acid residues 629–687) that connects the two homologous halves. The linker region contains four serine residues (serine 661, serine 667, serine 671, and serine 683) that are phosphorylated by PKC and/or PKA *in vitro* (Chambers *et al.*, 1993, 1994). Adapted from Gottesman and Pastan (1993).

(e.g., Garman *et al.*, 1983; Roy and Horwitz, 1985; Hamada *et al.*, 1987; Richert *et al.*, 1988; Schurr *et al.*, 1989; Meyers, 1989; Chambers *et al.*, 1990a; Ma *et al.*, 1991; Yu *et al.*, 1991; Bates *et al.*, 1992). Recombinant P-glycoproteins expressed in *mdr*-transfected mammalian cells (Schurr *et al.*, 1989) or in *MDR*1 baculovirus-infected insect cells (Germann *et al.*, 1990) are also phosphorylated. Center and colleagues (Center, 1983, 1985; Garman *et al.*, 1983) and Hamada *et al.* (1987) reported that enhanced drug sensitivity resulting from treatment of multidrug resistant cells with certain chemosensitizers (verapamil, trifluoperazine) or *N*-ethylmaleimide was associated with increased phosphorylation of P-glycoprotein. These findings led to the hypothesis that the drug efflux function of the multidrug transporter may be regulated by phosphorylation/dephosphorylation mechanisms.

During the past few years, many research groups have attempted to correlate changes of activities and/or levels of multiple protein kinases with levels of mdr and/or accumulation of drugs. Different cell lines in which P-glycoprotein is chiefly responsible for mdr were studied as model systems to analyze short-term effects of protein kinase activators and/or inhibitors on drug accumulation, or long-term effects on drug survival. Several laboratories have demonstrated that exposure of multidrug-resistant cells to activators of protein kinase C (PKC), such as the phorbol ester tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA) or 4β-phorbol-12β-myristate-13α-acetate (PMA), stimulated phosphorylation of P-glycoprotein, reduced drug accumulation, and enhanced drug resistance (e.g., Hamada *et al.*, 1987; Fine *et al.*, 1988; Chambers *et al.*, 1990a,b; Aftab *et al.*, 1994), although not necessarily for all cytotoxic agents tested (Bates *et al.*, 1992). Conversely, treatment of multidrug-resistant cells with protein kinase inhibitors (e.g., staurosporine, calphostin C, and the alkyl-lysophospholipid 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine) decreased phosphorylation of P-glycoprotein and increased drug accumulation (Ma *et al.*, 1991; Chambers *et al.*, 1992; Bates *et al.*, 1993). These observations seem to support the hypothesis that the state of phosphorylation of P-glycoprotein may regulate its drug efflux activity and, thus, modulate mdr. In agreement with this hypothesis, several protein kinase inhibitors (including staurosporine, H-87, H9, K525a) have been found to partially reverse multidrug resistance (O'Brian *et al.*, 1991; Miyamoto *et al.*, 1990, 1993a,b), or to enhance sensitization of cells to cytotoxic agents when applied together with a known mdr reversing agent such as verapamil (Sampson *et al.*, 1993b).

Protein kinase activators and inhibitors obviously affect the degree of mdr, but their molecular mechanisms of action are quite controversial. Most of the reagents used are not very specific and may have several effects in intact cells, which makes interpretation of the data difficult. While brief exposure of mammalian cells to the phorbol ester tumor promotor TPA results in PKC activation, prolonged exposure downregulates levels of PKC by increasing its rate of proteolysis (Young *et al.*, 1987; Nishizuka, 1988; Borner *et al.*, 1989; Huang *et al.*, 1989). Agonists of PKC (including TPA and DAG) may also increase *MDR*1 gene expression, both at the mRNA and protein level, as has been reported for normal peripheral blood cells and various cell lines derived from leukemias and solid tumors (Chaudhary and Roninson, 1992). This TPA- or DAG-induced activation of *MDR*1 gene expression could be suppressed with the protein kinase inhibitor staurosporine (Chaudhary and Roninson, 1992), suggesting that a PKC-mediated signal transduction pathway may be involved in upregulation of *MDR*1 gene expression. Similarly, various protein kinase antagonists may also alter *MDR*1 gene expression. Transcriptional activation of the *MDR*1 gene was found to be attenuated by the PKC inhibitor H7 (Uchiumi *et al.*, 1993), H-87, an inhibitor selective for cAMP-dependent protein kinase (PKA), reduced *MDR*1 gene transcription (Kim *et al.*, 1993), and low levels of staurosporine (1-7.5 nM), a nonselective protein kinase inhibitor, diminished P-glycoprotein expression levels in KB-V1 cells significantly, with a concomitant, but less pronounced decrease in *MDR*1 mRNA (Sampson *et al.*, 1993b). Under certain circumstances, however, staurosporine, when used alone, enhanced *MDR*1 gene expression (Chaudhary and Roninson, 1992; Sampson *et al.*, 1993b). Staurosporine, as well as many other hydrophobic protein kinase modulators, may also bind to the multidrug transporter upon entry into the cell and inhibit P-glycoprotein-mediated drug efflux by competing with drug substrates for transport (Sato *et al.*, 1990; Miyamoto *et al.*, 1992; Wakusawa *et al.*, 1992, 1993; Lelong *et al.*, 1994). Hence, protein kinase modulators may alter drug accumulation within multidrug-resistant cells independent of, or in addition to, their effects on the phosphorylation state of P-glycoprotein by interacting with the multidrug transporter directly, or by affecting levels of *MDR*1 gene expression. Activation of P-glycoprotein

expression may also underlie the observations that phorbol ester treatment of certain drug-sensitive human cell lines, lacking P-glycoprotein before treatment, can lead to reduced drug accumulation and the acquisition of a phenotype resembling mdr (O'Connor, 1985; Ido *et al.*, 1987; Ferguson and Cheng, 1987; Fine *et al.*, 1988).

PROTEIN KINASES THAT MAY PHOSPHORYLATE P-GLYCOPROTEIN

Several different protein kinases have been associated with phosphorylation of P-glycoprotein, including PKC (Chambers *et al.*, 1990b, 1992), PKA (Mellado and Horwitz, 1987), and at least three different novel kinases that remain to be identified (Staats *et al.*, 1990; Sampson *et al.*, 1993a; S. V. Ambudkar, I. H. Lelong, I. Pastan, M. M. Gottesman, unpublished).

Of the several potentially important protein kinases, PKC has received most attention. Using plasma membrane preparations from multidrug-resistant KB-V1 cells, partially purified P-glycoprotein, or a synthetic P-glycoprotein peptide, Chambers *et al.* (1990b, 1993, 1994) demonstrated that the human *MDR*1 gene product is phosphorylated by PKC *in vitro*. More recent studies using immunoprecipitated or chromatographically purified P-glycoprotein confirmed that it is a target for phosphorylation by PKC (Aftab *et al.*, 1994; S. V. Ambudkar, unpublished). As already discussed, stimulation of PKC activity by TPA can enhance phosphorylation of P-glycoprotein, reduce drug accumulation, increase drug transport, and elevate levels of drug resistance (Fine *et al.*, 1988; Chambers *et al.*, 1990a). Moreover, the mdr phenotype of cells obtained after multiple steps of cytotoxic selection is frequently, but not always, associated with elevated levels of PKC (Palayoor *et al.*, 1987; Fine *et al.*, 1988; Melloni *et al.*, 1989; O'Brian *et al.*, 1989; Posada *et al.*, 1989a,b; Aquino *et al.*, 1988, 1990).

PKC represents a family of related phospholipid-dependent isoenzymes which can be divided into three subfamilies: classical or conventional PKCs (PKC α, βI, βII, and γ), novel PKCs (including PKC δ, ϵ, and θ) and atypical PKCs (ξ and λ) (Nishizuka, 1992). Classical PKCs are calcium-dependent, whereas novel PKCs are calcium-independent, but members of both subfamilies are activated by phorbol ester or diacylglycerol. Cofactor requirements for atypical PKCs, have yet to be determined, although it has been established that PKCξ is not activated by phorbol esters (Nakanishi *et al.*, 1993). Our insights concerning the expression and the roles of the individual PKC isoenzymes in the mdr phenotype is rather limited at present, and very few studies have addressed the calcium-independent PKC isoenzymes (Blobe *et al.*, 1993; Bates *et al.*, 1993). Analyses of expression of calcium-dependent PKC isoenzymes in various multidrug-resistant cell lines has indicated selective upregulation of PKCα (Lee *et al.*, 1992; Posada *et al.*, 1989b; Yu *et al.*, 1991; O'Brian *et al.*, 1991; Gollapudi *et al.*, 1992; Blobe *et al.*, 1993), PKCβI (Fan *et al.*, 1992; Gollapudi *et al.*, 1992), or PKCγ (Aquino *et al.*, 1990). Transfection experiments have corroborated that overexpression of PKC isoenzymes in some P-glycoprotein-expressing cells may result in increased levels of mdr. PKCα (Yu *et al.*, 1991), but not PKCγ (Ahmad *et al.*, 1992), was found to increase mdr, decrease drug accumulation, and increase phorbol ester-stimulated phosphorylation of P-glycoprotein. In contrast, transfection of MCF-7/ADR cells with an expression vector containing a cDNA for PKCα in the anti-sense orientation reduced levels of PKCα, decreased total PKC activity, and diminished phosphorylation of P-glycoprotein, while increasing drug accumulation and drug sensitivity (Ahmad and Glazer, 1993). Thus, for PKCα there is good evidence that it may be involved in modulating mdr, but the exact mechanism for this effect has not been determined. A recent study by Ahmad *et al.* (1994) made use of an *MDR*1- and PKCα-baculovirus co-expression system to demonstrate a functional interaction of these two gene products. In membrane vesicles containing high amounts of both proteins, PKCα underwent autophyosphorylation and also phosphorylated P-glycoprotein. In the presence of PKCα, the basal vanadate-sensitive ATPase activity of wild-type P-glycoprotein was stimulated, in contrast to the ATPase activity of a mutant of P-glycoprotein in which a potential phosphorylation site was abolished. PKCα also increased substrate binding to P-glycoprotein slightly, as measured by azidopine photoaffinity labeling. Thus, in this insect cell expression system with high levels of both proteins in a membrane-associated form, PKCα appears to serve as a positive regulator of P-glycoprotein functions. These studies leave unresolved if phosphorylation by PKCα is also involved in controlling the activity of P-glycoprotein in intact mammalian cells.

The number of studies involving PKA is much more limited. The PKA enzyme in its holomeric form

consists of four subunits, two regulatory and two catalytic subunits. There are at least two different types of PKA that contain either regulatory subunit RI or regulatory subunit RII, but many more subtypes may exist (Francis and Corbin, 1994). Mellado and Horwitz (1987) demonstrated that phosphorylation of mouse P-glycoprotein is enhanced by cAMP and that P-glycoprotein is phosphorylated by the catalytic subunit of PKA. PKA may also phosphorylate human P-glycoprotein (Chambers *et al.*, 1994), but it is unknown if this might contribute to regulating levels of mdr. Similar to PKC, several studies suggest that PKA may also be involved in regulating *mdr* gene expression. As described earlier, the PKA inhibitor H-87 decreased *MDR*1 gene transcription (Kim *et al.*, 1993). Abraham *et al.* (1990) focused on the study of mutants of PKA that harbor altered RI regulatory subunits. Transfection of a mouse gene encoding a dominant negative mutant RI subunit into wild-type Chinese hamster ovary cells (CHO) abrogated the normal response of cells to cAMP stimuli, reduced cAMP stimulation of PKA activity, and rendered the cells more sensitive to various cytotoxic drugs that are substrates for the multidrug transporter. Similar characteristics were also observed for other CHO cells or mouse adrenal Y1 cells harboring PKA mutants (Abraham *et al.*, 1985, 1987; Chin *et al.*, 1992). Generally, the expression of the mutant RI gene was associated with a decrease in the expression of *mdr* mRNA and gene product, suggesting that in these cells normal PKA activity is required to sustain basal levels of P-glycoprotein (Abraham *et al.*, 1990; Chin *et al.*, 1992).

Other protein kinases that may be involved in regulating P-glycoprotein expression and/or functional activity are less well characterized and include a novel protein kinase of unknown molecular weight, discovered by Staats *et al.* (1990). Its properties were similar to protein kinase P (Elias and Davis, 1985), a protein kinase of 27 kDa in platelets. It represents a membrane-associated kinase occurring both in drug-sensitive and drug-resistant HL60 cells that is phospholipid-dependent, but calcium-independent, and capable of phosphorylating P-glycoproteins *in vitro* (Staats *et al.*, 1990). Two additional membrane-bound kinases that may play a role in modulating mdr have been detected in multidrug resistant KB-V1 cells (Sampson *et al.*, 1993a; S. V. Ambudkar, G. Park, C. O. Cardarelli, I. Pastan, and M. M. Gottesman, unpublished). One of them, a 170-kDa serine/threonine protein kinase present in multidrug-resistant KB-V1 cells, but not in parental KB-3-1 cells, exhibits a pattern of expression in certain cells that correlates with levels of mdr (Sampson *et al.*, 1993). This 170-kDa protein kinase is not dependent on calcium, phosphatidylserine, or cAMP, but is affected by kinase inhibitors staurosporine, K525a, and KT5720. Phosphorylation of P-glycoprotein by the 170-kDa protein kinase has not been demonstrated and substrate(s) are unknown at present. Recently, a 55–60-kDa protein kinase was purified from plasma membrane preparations isolated from multidrug-resistant KB-V1 cells that is also present in the drug-sensitive KB-3-1 parental cell line (S. V. Ambudkar, G. Park, C. O. Cardarelli, I. Pastan, and M. M. Gottesman, unpublished). This protein kinase is referred to as "V-1 kinase." It phosphorylates P-glycoprotein *in vitro*, but not histone, or PKC and PKA peptide substrates. Its kinase activity requires either Mg^{2+} or Mn^{2+}, is sensitive to >1 mM Ca^{2+}, and is inhibitable by staurosporine.

In vesicles from multidrug-resistant cells which contain P-glycoprotein and are capable of ATP-dependent drug transport, ATP stimulates phosphorylation of P-glycoprotein in the presence of inhibitors of PKC and PKA (Lelong *et al.*, 1994). The extent of phosphorylation is stimulated severalfold by GTP, suggesting a role for this nucleotide in activating a kinase, inhibiting phosphatases, or changing ATP levels (Lelong *et al.*, 1994).

At present, the individual contribution of the different kinase activities described above to the overall levels of P-glycoprotein and its state of phosphorylation in a given cell line are not clearly established. It appears that depending on the species, cell type, and/or state of differentiation multiple kinases may contribute to the extent of phosphorylation of P-glycoprotein, and/or may regulate its activity. Moreover, the types of phosphatases that dephosphorylate P-glycoprotein remain to be identified. Preliminary data suggest an involvement of the membrane-associated protein phosphatases 1 and/or 2A (Chambers *et al.*, 1992), but additional studies are required to define the enzymology and kinetics of the phosphorylation/dephosphorylation cycle of P-glycoprotein.

IDENTIFICATION OF PHOSPHORYLATION SITES WITHIN P-GLYCOPROTEIN

Recent approaches to assess the role of phosphorylation in P-glycoprotein-mediated mdr have focused on the identification of actual sites of

phosphorylation within the multidrug transporter to provide a biochemical basis for site-directed mutagenesis. In early studies, phosphoamino acid analysis of P-glycoprotein was performed (Center, 1983; Roy and Horwitz, 1985; Hamada *et al.*, 1987). Consistently, phosphoserine, but no phosphotyrosine, was detected. For mouse P-glycoprotein, both phosphoserine and phosphothreonine were observed, but recent studies suggest the presence of phosphoserine exclusively (Orr *et al.*, 1993). The work of several investigators suggested that more than one site can be phosphorylated and that different kinases are able to do this (Mellado and Horwitz, 1987; Fine *et al.*, 1988; Chambers *et al.*, 1990b, 1994; Staats *et al.*, 1990). Inspection of the primary sequence of P-glycoprotein indicates the presence of numerous (> 40) consensus sites for phosphorylation by PKC or PKA (Kenelly and Krebs, 1991) distributed throughout the length of the molecule. But according to studies by Chambers *et al.* (1993, 1994) for human P-glycoprotein and by Orr *et al.* (1993) for mouse P-glycoprotein, a central region that connects the two homologous halves of P-glycoprotein harbors the major sites of phosphorylation by PKC and PKA (see Fig. 1). This region represents a cytosolic segment of approximately sixty amino acid residues and is commonly referred to as the linker region. A comparison of the deduced amino acid sequences of the linker region from a variety of mammalian drug-transporting P-glycoproteins (Fig. 2) reveals that the linker region exhibits a characteristic high content (34–40%) of charged amino acids (Fig. 2). Similarly, a relatively high content of consensus sequences for phosphorylation by PKC and PKA, requiring basic amino acids near the phosphoacceptor group, is noticeable.

Two-dimensional tryptic phosphopeptide maps obtained from human P-glycoprotein that was metabolically labeled *in vivo* suggested the presence of at least three major sites of phosphorylation (Chambers *et al.*, 1992). *In vitro* experiments using human P-glycoprotein present in plasma membrane vesicles as a substrate for phosphorylation by PKC gave the same two-dimensional tryptic phosphopeptide maps as observed in the *in vivo* phosphorylation studies (Chambers *et al.*, 1993). Amino acid sequence analysis of isolated P-glycoprotein phosphopeptides identified serine 661, serine 671, and one or more of serine 667, serine 675, or serine 683 as sites of phosphorylation (Chambers *et al.*, 1993). Studies involving a synthetic peptide encompassing amino acid residues 656–689 of the human P-glycoprotein linker region as target for *in vitro* phosphorylation confirmed serine 661 and serine 671 as PKC sites, and revealed serine 667 as a third PKC site (Chambers *et al.*, 1994). The synthetic linker peptide was also used as a substrate for *in vitro phosphorylation* by PKA, and serine 667, serine 671, and serine 683 were phosphorylated by this enzyme. Similar experiments were performed with "V-1 kinase" which appears to phosphorylate serine 661 and serine 667 (S. V. Ambudkar and T. C. Chambers, unpublished). Recently, a glutathione-*S*-transferase (GST) fusion approach was used to express and purify a series of GST-P-glycoprotein fusion proteins containing a major portion of the linker region (amino acid residues 644–689) of the human *MDR*1 gene product (T. C. Chambers, U. A. Germann, M. J., Gottesman, I., Pastan, J. F. Kuo, and S. V. Ambudkar, unpublished). These fusion proteins were subjected to *in vitro* phosphorylation by PKC and "V-1 kinase," and tryptic phosphopeptides were analyzed by two-dimensional

Hu MDR1 633 NEVELENAADESKSEIDALEMSSNDSRS**S**LIRKR**S**TRR**S**VRGSQAQDRKL**S**TKEALDESI 692

Ha pgp1 630 NEIELGNEVGESKNEIDNLDMSSKDSASSLIRRR**S**TRR**S**IRGPHDQDRKL**S**TKEALDEDV 689

Mu mdr1 629 NEIELGNEACKSKDEIDNLDMSSKDSGSSLIRRR**S**TRKSICGPHDQDRKL**S**TKEALDEDV 688

Ha pgp2 632 NEVELGSEADGSQSDTIASELTSEEFKSP**S**VRK-**S**TCRSICGSQDQERRV**S**VKEAQDEDV 690

Mu mdr1 632 NEIEPGNNAYGSQSDTDASELTSEESKSPLIR-R**S**IYR**S**VHRKQDQERRL**S**MKEAVDEDV 690

Hu MDR2 635 SQIQSEEF--ELNDEKAATRMAPNGWKSRLFRH**S**TQKNLKNSQMCQKSLDVETDGLEANV 692

Mu mdr2 632 SQILSEEFEVELSDEKAAGDVAPNGWKARIFRN**S**TKK**S**LK-SPH-QNRLDEETNELDANV 689

Fig. 2. Alignment of deduced amino acid sequences of P-glycoprotein linker regions and predicted PKC phosphorylation sites. Sequences from drug-transporting P-glycoproteins (top five sequences) are separated from sequences of non-drug-transporting P-glycoproteins (bottom two sequences). Serine residues occurring within PKC consensus sequences {(R/K_{1-3}) (X_{2-0})–S–(X_{2-0}) (R/K_{1-3}), except where both X's are zero} are highlighted and underlined.

mapping. A mutational analysis was performed in which putative phosphorylation sites were substituted systematically with nonphosphorylatable alanine residues. These experiments corroborated that serine 661 and serine 667 are targets for *in vitro* phosphorylation by PKC. Serine 683 was also phosphorylated by PKC *in vitro*, but only in the absence of all other putative phosphorylation sites. Serine 661 and serine 667, but not serine 683, were also phosphorylated by the "V-1 kinase" *in vitro*. Despite the presence of other potential phosphorylation sites within the linker region of human P-glycoprotein (including other serine residues, as well as threonine residues), a cluster of maximally four serine residues appears to be accessible and recognized by various protein kinases (summarized in Fig. 1). Taken together, these studies demonstrate that the linker region within the human *MDR*1 gene product represents the preferred target for multisite phosphorylation by several protein kinases. Similarly, two serine residues present within the linker region of the mouse *mdr*1b P-glycoprotein are phosphorylated *in vitro*, namely serine 669 by PKC (analogous to serine 671 in the human *MDR*1 gene product), and serine 681 by PKA (analogous to serine 683 in the human *MDR*1 gene product) (Orr *et al.*, 1993). Several PKC and/or PKA consensus phosphorylation sites are also found in the linker region of the mouse *mdr*3 and the hamster *pgp*1 P-glycoproteins (Fig. 2), but the actual sites of phosphorylation have not been identified, to date.

It is interesting to note that the linker region of human P-glycoprotein is present at an analogous location within the polypeptide chain as a putative regulatory domain of another ABC transporter, the R-domain of the cystic fibrosis transmembrane conductance regulator (CFTR). The R-domain of CFTR contains a large number of potential phosphorylation sites, some of which contribute to the regulation of its cAMP-dependent chloride channel function (Riordan *et al.*, 1989; Cheng *et al.*, 1991, 1993). In analogy with the R-domain of the CFTR protein, the linker region of P-glycoprotein has been hypothesized to represent a "mini-R-domain" that may function to regulate its biological activity (Chambers *et al.*, 1993).

MUTATIONAL ANALYSIS OF P-GLYCOPROTEIN PHOSPHORYLATION SITES

There is ample evidence that P-glycoprotein is phosphorylated *in vivo*, and many studies suggest that protein kinases may play a role in modulating mdr. It has been difficult, however, to clearly establish the exact role of phosphorylation of P-glycoprotein in regulating its multidrug transporter activity. The identification of the major sites of phosphorylation within P-glycoprotein provides an opportunity to address this problem by site-directed mutagenesis. Recently, we have initiated a mutational analysis of the putative phosphorylation sites within the linker region by substituting various serine residues with either nonphosphorylatable alanine residues, or aspartic acid residues to mimic permanently phosphorylated serine residues (U. A. Germann, T. C. Chambers, S. V. Ambudkar, T. Licht, C. O. Cardarelli, I. Pastan, and M. M. Gottesman, unpublished). Our data confirm that the major phosphorylation sites are confined to the linker region of P-glycoprotein. Moreover, the phosphorylation- and dephosphorylation-defective P-glycoprotein mutants are expressed at the cell surface and are able to confer an mdr phenotype to drug-sensitive cells similar to wildtype P-glycoprotein. Thus, multidrug resistance can be mediated by P-glycoprotein without detectable levels of phosphorylation, or in a stage of permanent phosphorylation. While phosphorylation of P-glycoprotein may not be required for its basal drug efflux activity, our data do not rule out the possibility that phosphorylation may modulate the mdr phenotype conferred by P-glycoprotein. Stable transfectants were obtained that produce large quantities of the phosphorylation-defective mutants of P-glycoprotein, but we do not know, yet, if their half-life is the same or different from that of wild-type P-glycoprotein. It is also possible that phosphorylation of P-glycoprotein may somewhat affect the specificity of drug transport and/or its response to inhibitors. Alternatively, phosphorylation may influence the kinetics of the drug transport. Experiments using purified phosphorylation-defective mutants and wild-type P-glycoprotein that are functionally reconstituted in phospholipid vesicles (see S. V. Ambudkar *et al.*, this issue) may be necessary to provide answers to these questions. Most intriguingly, however, it remains a possibility that phosphorylation of the *MDR*1 gene product may be less important for drug efflux, but may be essential for the transport of a putative physiologic and substrate(s) that still remain(s) to be identified.

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