Bacterial Expression of the Linker Region of Human *MDR*1 P-Glycoprotein and Mutational Analysis of Phosphorylation Sites†

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ABSTRACT: Phosphorylation may play a role in modulating multidrug resistance by P-glycoprotein (P-gp). The linker region between the two homologous halves of human P-gp harbors several serine residues which are phosphorylated by protein kinase C (PKC) *in vitro*. We used the glutathione *S*-transferase gene fusion system to express and purify a series of fusion proteins containing the relevant portion (residues 644−689) of the linker region of the human *MDR*1 gene product. The fusion proteins were subjected to *in vitro* phosphorylation and phosphopeptide mapping analysis to identify specific phosphorylation sites. On the basis of a mutational strategy in which individual serine residues were systematically replaced with nonphosphorylatable alanine residues, Ser-661 and Ser-667 were identified as major PKC sites and Ser-683 was identified as a minor PKC site. Ser-661 and Ser-667 were also found to be the primary sites of phosphorylation for a novel membrane-associated P-gp specific kinase isolated from the multidrug-resistant KB-V1 cell line. Individual phosphorylation sites were recognized independently of each other. These data show that the linker region of P-gp represents a target for multisite phosphorylation not only for PKC but also for the P-gp specific V1 kinase. Specific serine phosphorylation sites are identified, and evidence is presented that the V1 kinase has a specificity which overlaps, but is more restricted than, that of PKC. In addition, these studies also suggest that the use of GST fusion peptides may be applicable for the analysis of multisite and ordered protein phosphorylation in other systems.

Overexpression of P-glycoprotein (P-gp),[1] an energy-dependent drug efflux pump of broad specificity, confers resistance to multiple, structurally unrelated anticancer drugs and other hydrophobic compounds due to decreased intracellular accumulation of these agents [reviewed in Gottesman and Pastan (1993)]. It has been demonstrated in a wide variety of MDR cell lines that P-gp molecules undergo covalent modification by phosphorylation [reviewed in Glazer (1994); Germann et al. (1995)]. However, the extent to which phosphorylation may modulate the activities ascribed to P-gp, which include drug transport (Horio et al., 1988), drug binding (Cornwell et al., 1986), ATPase activity (Sarkadi et al., 1992; Ambudkar et al., 1992), and perhaps chloride (Gill et al., 1992) and ATP (Abraham et al., 1993) channel activities, is not well understood. On the basis of studies of drug accumulation and drug efflux in response to protein kinase activators and/or inhibitors, there is some evidence that phosphorylation stimulates P-gp-mediated transport of certain drugs. The PKC activator TPA, for example, has been shown to increase Pgp phosphorylation and reduce net drug accumulation in a variety of MDR cell lines (Fine et al., 1988; Yu et al., 1991; Chambers et al., 1992; Bates et al., 1993). Conversely, the protein kinase inhibitors staurosporine and calphostin C are effective inhibitors of P-gp phosphorylation and cause an increase in net drug accumulation in MDR, but not drug-sensitive, cells (Ma et al., 1991; Chambers et al., 1992; Bates et al., 1993; Aftab et al., 1994). These results should be interpreted with caution, however, because TPA can rapidly increase P-gp mRNA levels in certain cells (Chaudhary & Roninson, 1992), and staurosporine and other kinase inhibitors may directly interact with P-gp and thus compete with drugs for transport [reviewed in Germann et al. (1995)].

In order to develop new approaches to investigate the role of phosphorylation in P-gp function, recent work has focused on the identification of the phosphorylation sites. The major sites occur in the linker region which contains several PKC/PKA consensus sequences (Glazer, 1994; Germann et al., 1995). In human *MDR*1 P-gp, two *in vitro* PKC sites, Ser-661 and Ser-671, have been identified (Chambers et al., 1993), and a third site, Ser-667, was inferred from analysis of a relevant synthetic peptide substrate (Chambers et al., 1994). Recently, a single PKC site, Ser-669, and a single PKA site, Ser-681, have been identified in the linker region

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[1] Abbreviations: P-gp, P-glycoprotein; MDR, multidrug-resistant or multidrug resistance; PKC, protein kinase C; PKA, cyclic AMP-dependent protein kinase; TPA, 12-0-tetradecanoylphorbol-13-*O*-acetate; GST, glutathione *S*-transferase; V1 kinase, P-gp specific protein kinase isolated from KB-V1 cell membrane; PL, P-gp linker; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

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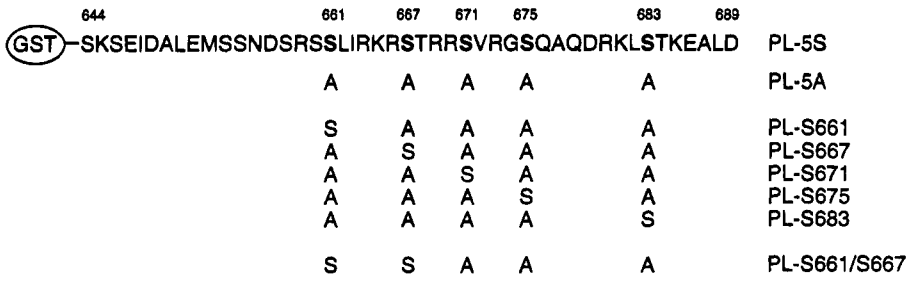

FIGURE 1: Schematic representation and nomenclature of GST fusion proteins. The wild-type sequence of residues 644−689 from the linker region of *MDR*1 P-gp is shown at the top and the five serines (positions 661, 667, 671, 675, and 683) selected for substitution are indicated. The sequences and nomenclature of the alanine mutant constructs with systematic Ser to Ala replacements are shown beneath.

of mouse *mdr*1b P-gp (Orr et al., 1993). Several PKC/PKA consensus phosphorylation sites are also present in mouse *mdr*1a P-gp (Devault & Gros, 1990) and hamster *pgp*1 (Devine et al., 1991), but the actual sites of phosphorylation in these P-gps have not yet been reported.

As a means to identify and mutate specific phosphorylation sites, we describe in this paper the use of a bacterial expression system involving glutathione *S*-transferase (GST) fusion proteins containing the relevant portion of the linker region of *MDR*1 P-gp. Thus, a series of fusion proteins with defined Ser to Ala replacements at the PKC consensus sites of the inserted P-gp sequence were constructed, expressed in *Escherichia coli* and purified. The fusion proteins were tested and compared as substrates for rat brain PKC and for a purified P-gp specific protein kinase, isolated from KB-V1 cell membranes.[2] Through this approach we were able to unambiguously confirm the location of the major PKC sites and identify the specific sites phosphorylated by the V1 kinase. The two kinase preparations exhibited a very similiar specificity suggesting that the P-gp kinase is closely related to PKC family members. We also present evidence that two major sites are phosphorylated in an ordered, or hierarchical, fashion. The expression of these modified cDNAs and their characterization provides a firm experimental basis and the necessary methodology for the construction of a series of full-length cDNAs encoding well-defined phosphorylation site mutants of P-gp.

EXPERIMENTAL PROCEDURES

Materials. The pGEX-2T gene fusion vector and glutathione Sepharose 4B beads were obtained from Pharmacia LKB Biotechnology, Inc. Purified rat brain PKC, comprising a mixture of α, β, and γ isoforms, was purchased from Calbiochem. [γ-^{32}P]ATP (6000 Ci/mmol) was either from ICN Radiochemicals or Dupont NEN. Troponin I was purified from bovine heart as described previously (Potter, 1982).

Construction, Expression, and Isolation of GST Fusion Proteins. Small cDNA fragments encompassing nucleotides 1930−2070 of the human *MDR*1 open reading frame, encoding residues 644−689 of the linker region of P-gp (Chen et al., 1986) followed by a translation stop codon, were generated with either the wild-type sequence or with specific Ser to Ala substitutions at the consensus PKC sites (see Figure 1), and fused in frame with the GST gene in the pGEX-2T expression vector. To this end, a series of oligodeoxynucleotides were synthesized (BioServe Biotechnologies, Laurel, MD) including UAG-84 (5′-GATCCAAAAGTGAAATCGATGCCTTGGAAAT-3′), UAG-85 (5′-TCATTTGAAGACATTTCCAAGGCATCGATTTCACTTTTG-3′), UAG-86 (5′-GTCTTCAAATGATTCAAGATCCAGTCTA-3′), UAG-87 (5′-TCTTTTTCTTATTAGACTGGATCTTGAA-3′), UAG-88 (5′-ATAAGAAAAAGATCAACTCGTAGGAGTGTCCGTGGATCA-3′), UAG-89 (5′- GTCTTGGGCTTGTGATCCACGGACACTCCTACGAGTTGA-3′), UAG-90 (5′-CAAGCCCAAGACAGAAAGCTTAGTACCAAAGAGGCTCTAGATTG-3′), and UAG-91 (5′-AATTCAATCTAGAGCCTCTTTGGTACTAAGCTTTCT-3′) to generate the wild-type PL sequence, and UAG-92 (5′-GTCTTCAAATGATTCAAGATCCGCTCTA-3′), UAG-93 (5′-TCTTTTTCTTATTAGAGCGGATCTTGAA-3′), UAG-94 (5′-ATAAGAAAAAGAGCAACTCGTAGGGCTGTCCGTGGAGCA-3′), UAG-95 (5′-GTCTTGGGCTTGTGCTCCACGGACAGCCCTACGAGTTGC-3′), UAG-96 (5′-CAAGCCCAAGACAGAAAGCTTGCTACCAAAGAGGCTCTAGATTG-3′), UAG-97 (5′-AATTCAATCTAGAGCCTCTTTGGTAGCAAGTTCTCT-3′), UAG-98 (5′-ATAAGAAAAAGATCAACTCGTAGGGCTGTCCGTGGAGCA-3′), UAG-99 (5′-GTCTTGGGCTTGTGCTCCACGGACAGCCCTACGAGTAGT-3′), UAG-100 (5′-ATAAGAAAAAGAGCAACTCGTAGGAGTGTCCGTGGAGCA-3′), UAG-101 (5′-GTCTTGGGCTTGTGCTCCACGGACACTCCTACGAGTTGC-3′), UAG-102 (5′-ATAAGAAAAAGAGCAACTCGTAGGGCTGTCCGTGGATCA-3′), UAG-103 (5′-GTCTTGGGCTTGTGATCCACGGACAGCCCTACGAGTTGC-3′) to generate the various P-glycoprotein linker (PL) mutants. All oligodeoxynucleotides were purified by denaturing PAGE and subsequently phosphorylated at the 5′ end (with the exception of UAG-84, -91, and -97) using T4 polynucleotide kinase as described (Sambrook et al., 1989). Equimolar amounts of four pairs of complementary and overlapping oligodeoxynucleotides were then annealed in the presence of 10 mM $MgCl_2$ by heating the mixture to 80 °C and cooling slowly to room temperature. Mixtures for expression of the P-gp mutants included UAG-84, -85, and UAG-92−97 for PL-5A; UAG-84−87 and UAG-94−97 for PL-S661; UAG-84, -85, -92, -93, and UAG-96−99 for PL-S667; UAG-84, -85, -92, -93, -96, -97, -100, and -101 for PL-S671; UAG-84, -85, -92, -93, -96, -97, -102, -103 for PL-S675; UAG-84, -85 and UAG-90−95 for PL-S683; and UAG-84−87 and UAG-96−99 for PL-S661/S667; all representing *Bam*HI−*Eco*RI fragments. To construct the various expression vectors, aliquots of annealed oligodeoxynucleotides were ligated with the pGEX-2T plasmid that had been linearized with *Bam*HI−*Eco*RI restriction enzymes and purified by agarose gel electrophoresis (Sambrook et al., 1989). Ligation mixes were used to transform competent

[2] S. V. Ambudkar, et al., manuscript in preparation.

E. coli DH5a (GIBCO-BRL, Gaithersburg, MD), and the presence and correctness of the various *MDR*1 inserts within the pGEX-2T derivatives was confirmed by restriction enzyme digestions and DNA sequence analysis (ABI automated sequencer) in both directions using oligodeoxynucleotides UAG-82 (5′-GCCTTTGCAGGGCTGGCAAGC-3′) and UAG-83 (5′-GAGGTTTTCACCGTCATCACC-3′) as primers. Bacteria were induced for expression with 0.1 mM isopropyl-β-D-thiogalactoside, cell lysates were prepared, and GST−P-gp peptide fusion proteins were purified using glutathione Sepharose 4B as described (Smith & Johnson, 1988). The proteins were concentrated (Amicon Centricon-10) to 0.1−1.0 mg/mL in 0.1 M Tris−HCl, pH 7.5, 0.12 M NaCl, 20 mM glutathione and stored at −70 °C. Proteins were resolved by 12% acrylamide SDS−PAGE (Laemmli, 1970), and Western blotting was performed after transfer to polyvinylidene difluoride membranes (Towbin et al., 1979). The membranes were incubated with the αPEPG-2 antibody (1:100 dilution) which recognizes the linker region of P-gp (Bruggemann et al., 1990). Bound antibody was detected by enhanced chemiluminescence (Amersham) with anti-rabbit IgG-linked peroxidase (1:500 dilution) as described by the manufacturer.

Preparation of V1 Kinase. The P-gp specific kinase, termed the V1 kinase, was purified from KB-V1 cell membranes. The purification scheme and characterization of the enzyme will be described in detail elsewhere.[2] Briefly, purified plasma membranes were solubilized with octylglucoside in the presence of lipid and glycerol, and the detergent extract was chromatographed on DEAE Sepharose CL-6B, as described (Ambudkar et al., 1992). A V1 kinase-enriched fraction eluting at 0.3 M NaCl was concentrated and chromatographed on a column of phosphocellulose (P-11, Whatman), and the kinase was eluted with 0.2 M NaCl. Further purification was achieved by gel filtration using Sephacryl S-200; the enzyme was eluted with an apparent molecular weight of 55 kDa. The degree of purification relative to the detergent extract was 1000- to 1500-fold. Partially purified human P-glycoprotein (Ambudkar et al., 1992) was used as a substrate to assay the V1 kinase. Enzyme activity was not affected by various PKC and PKA inhibitors, including H7, calphostin C, PKC inhibitor peptide-(19−36), PKA inhibitor peptide PKI-(6−22)-amide, or K-252b. However, it was inhibited by the nonspecific kinase inhibitor staurosporine at 1−2 μM. The V1 kinase failed to phosphorylate histone-IIIS, casein, PKC peptide-(19−31), and kemptide.

Phosphorylation of Fusion Proteins. Standard reaction mixtures of 0.1 mL contained 10 μg of fusion protein in 25 mM Tris−HCl, pH 7.5, 10 mM $MgCl_2$, and 10 μM [γ-^{32}P]-ATP (4×10^3 cpm/pmol). Phosphorylation with PKC (10 ng) was carried out in the presence of 0.5 mM $CaCl_2$ and 50 μg of phosphatidylserine/mL. Phosphorylation with V1 kinase (1−2 μg) was carried out in the presence of 5 mM $MnCl_2$. Reactions were performed for 30 min at 30 °C, and protein phosphorylation was analyzed by 12% acrylamide SDS−PAGE and autoradiography with Kodak XAR-1 film at −70 °C. Preparation of membrane vesicles from KB-V1 cells and phosphorylation of Pgp by PKC *in vitro* were performed as described previously (Ambudkar et al., 1992; Chambers et al., 1990).

Phosphopeptide Mapping and Phosphoamino Acid Analysis. Phosphorylated protein bands were excised from dried gels, minced, washed in water (5 × 1 mL), washed in 1 mL of 0.1 M NH_4HCO_3, pH 8.0, suspended in 0.5 mL of 0.1 M NH_4HCO_3, pH 8.0, and digested with 100 μg TPCK-treated trypsin (Sigma) for 24 h at 37 °C. The digestion mixture was lyophilized, and the peptides were dissolved in 20 μL of water and resolved in two dimensions on cellulose thin-layer plates as described (Chambers et al., 1992). Briefly, electrophoresis was for 3 h at 450 V toward the cathode in a solvent system of pyridine/glacial acetic acid/water, 1:10:89, pH 3.7. Chromatography was in *n*-butanol/pyridine/glacial acetic acid/water, 50:33:1:40. Phosphoamino acid analysis was performed as described previously (Chambers et al., 1993).

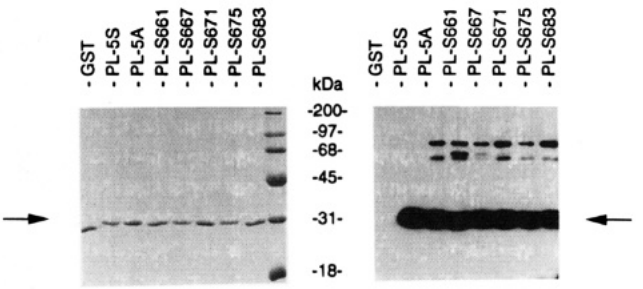

FIGURE 2: Analysis of fusion proteins. Left panel: Coomassie-stained 12% acrylamide SDS gel of the GST control and the GST fusion proteins (0.5 μg each). Right panel: Western blot of duplicate gel probed with αPEPG-2 antibody. Arrow indicates migration position of fusion proteins at ~31 kDa. Molecular mass standards (in kDa) are shown in the center.

RESULTS

The amino acid sequences of the wild-type and mutant constructs of PL peptides which were expressed as fusion proteins with GST are shown in Figure 1. Five serine residues at positions 661, 667, 671, 675, and 683 occur in PKC consensus sequences (Kennelly & Krebs, 1991), and these were chosen for substitution with nonphosphorylatable alanine. The construct with the wild-type sequence is termed PL-5S, and the construct with all five alanine substitutions is termed PL-5A. The constructs that systematically retain one of the five serines, with alanine substitutions at the other four positions, are termed PL-S661 through PL-S683, respectively. An additional construct termed PL-S661/S667 was made with two serines at positions 661 and 667 and alanines at the other three positions.

The fusion proteins were expressed in *E. coli*, purified, and analyzed by SDS−PAGE (Figure 2, left panel). They all exhibited similar electrophoretic mobilities of apparent molecular mass ~31 kDa; as expected, the mass of each was greater than that of the GST control. All of the fusion proteins, but not the GST control, reacted on a Western blot with αPEPG-2 antibody (Figure 2, right panel), which specifically recognizes the linker region of human *MDR*1 P-gp (Bruggemann et al., 1990), verifying that they each contain the expected P-gp sequence.

Before testing the fusion proteins as kinase substrates, it was necessary to determine whether GST itself was a substrate. Although certain mammalian GST isoforms are *in vitro* PKC substrates (Taniguchi & Pyerin, 1989), the *Schistosoma japonicum* parasite GST utilized here had not previously been tested in such an assay. We used troponin I, an effective PKC substrate (Noland et al., 1989), as a positive control. As shown in Figure 3, under conditions

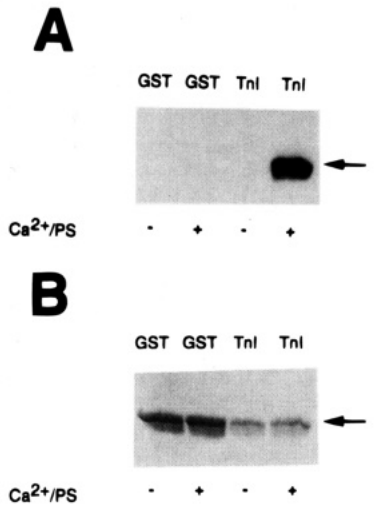

FIGURE 3: GST is not phosphorylated by PKC. GST or troponin I (TnI) (10 μg) was incubated in standard PKC assay mixtures for 30 min and analyzed by SDS−PAGE and autoradiography. Reactions were performed in the absence or presence of 0.5 mM $CaCl_2$ plus 50 μg of phosphatidylserine/mL (Ca^{++}/PS) as indicated. A, ^{32}P autoradiography; B, Coomassie stain of same gel. Troponin I and GST have similar apparent molecular weights of ~30 kDa.

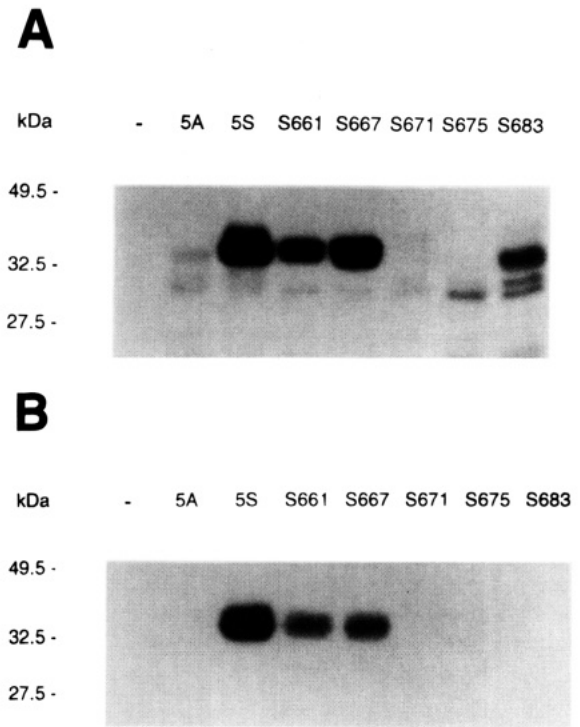

FIGURE 4: Phosphorylation of fusion proteins. A, phosphorylation by PKC; B, phosphorylation by V1 kinase. Autoradiographs are shown. See Experimental Procedures for details.

where troponin I was phosphorylated by PKC in a calcium- and phospholipid-dependent manner, no phosphorylation of GST was detected. Similarly, GST was not a substrate for the V1 kinase preparation (data not shown).

The fusion proteins were incubated for 30 min in a standard PKC assay mixture; analysis by SDS−PAGE and autoradiography indicated that PL-5S was phosphorylated whereas PL-5A showed only very faint labeling (Figure 4A). Among the single-serine constructs, PL-S661, PL-S667, and PL-S683 were phosphorylated by PKC whereas PL-S671 and PL-S675 were not (Figure 4A). The relative incorporation of phosphate was in the order PL-5S > PL-S667 > PL-S661 > PL-S683. When the fusion proteins were tested as substrates for the V1 kinase, PL-5S, PL-S661, and PL-S667 were phosphorylated whereas PL-5A and the other single serine constructs were not (Figure 4B). The relative incorporation of phosphate was PL-5S > PL-S667 = PL-S661. It is evident that with the exception of PL-S683, which was phosphorylated by PKC but not by the V1 kinase, the two kinase preparations exhibited a very similar specificity. Thus the substrate preference of the V1 kinase overlaps, but is more restricted than, that of PKC. It is also evident that both kinases failed to recognize the remaining serines (positions 644, 646, 654, 655, 658, 660) and two threonines (positions 668 and 684) present in PL-5A.

Further information on the relative specificities of the two kinases and identification of the sites of phosphorylation were obtained by phosphoamino acid analysis and phosphopeptide mapping. In preparations of PL-5S phosphorylated by either kinase, only phosphoserine and no phosphothreonine was detected (data not shown). Tryptic phosphopeptide mapping of PL-5S phosphorylated by the V1 kinase resolved two distinct phosphopeptide spots (Figure 5A). The same two phosphopeptides were detected upon analysis of PL-5S phosphorylated by PKC (Figure 5B). In view of this result, and because Ser-661 and Ser-667 were the only sites recognized in common by both kinases, on the basis of data with the single-serine constructs (Figure 4), we constructed PL-S661/S667 (see Figure 1). This double-serine construct was phosphorylated by the V1 kinase or PKC and subjected to phosphopeptide mapping (Figure 5C,D). The map pattern was found to be the same for both kinases and the same as that of PL-5S. We conclude that of the five PKC consensus sites in PL-5S, only two, Ser-661 and Ser-667, are phosphorylated by PKC, and phosphorylation by the V1 kinase similarly involves these two sites. Of the two phosphopeptides resolved, the upper one has previously been identified as that containing Ser-661 by amino acid sequencing (Chambers et al., 1993, 1994). In addition, digestion and analysis of phosphorylated PL-S661 yielded a single phosphopeptide with the same map coordinate (Figure 5E). By inference the lower phosphopeptide contains Ser-667.[3] On the basis of the distribution of phosphate between the two sites in PL-5S, PKC appears to preferentially phosphorylate Ser-661 (Figure 5B), whereas the V1 kinase appears to phosphorylate the two sites approximately equally (Figure 5A).

Phosphopeptide mapping of PL-683, which was phosphorylated only by PKC (Figure 4), was also performed (Figure 5F). In this case, the radioactivity was found to be distributed among three weakly labeled peptides whose migration positions were distinct from the peptides containing Ser-661 and Ser-667. The appearance of multiple phosphopeptides from PL-S683 may be due to the presence of products of partial trypsin digestion. Such a possibility would not be unexpected given the sequence (RKLSTKE) around this site. For example, trypsin digestion is often inhibited when the susceptible R/K bond is followed by a glutamic acid residue, and when basic amino acids occur in clusters of two or more

[3] This assignment is consistent also with the relative migration of the two phosphopeptides in the second dimension where more hydrophobic peptides migrate faster. The expected composition of the tryptic peptide containing Ser-661 is SSLIR and that containing Ser-667 is STR.

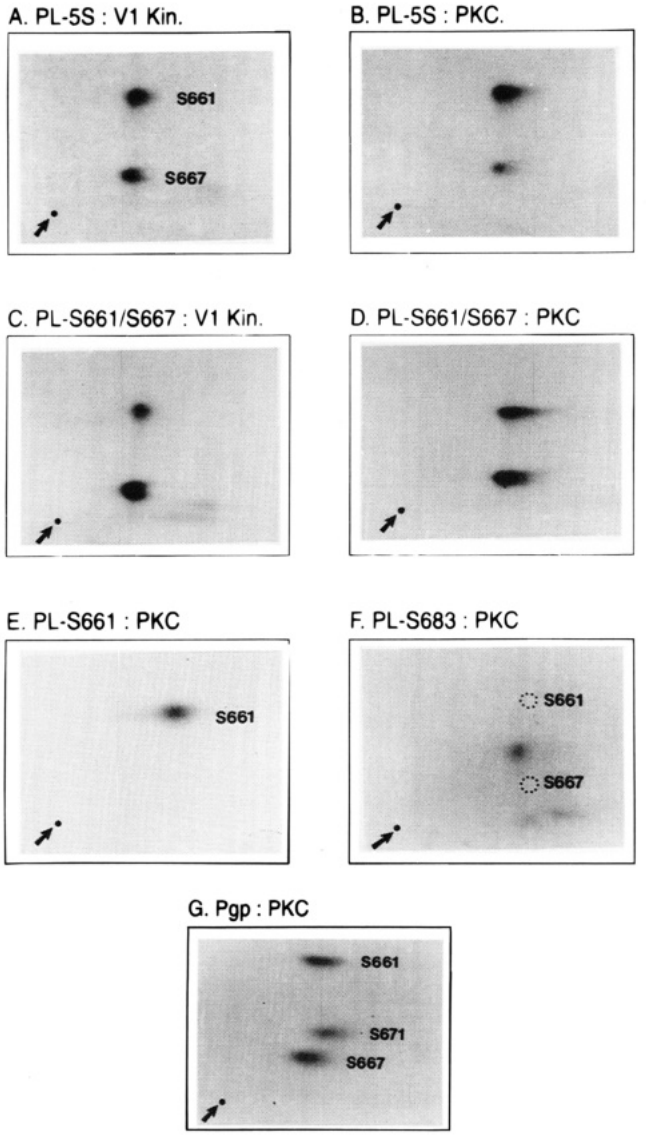

FIGURE 5: Autoradiographs of two-dimensional phosphopeptide maps. A−D, preparations of PL-5S or PL-S661/S667 were phosphorylated with V1 kinase (V1 Kin.) or PKC as indicated, and equivalent amounts of radioactivity (~2,500 cpm) were subjected to two-dimensional tryptic phosphopeptide mapping; E−G, phosphopeptide maps of PL-S661 (1050 cpm), PL-S683 (~400 cpm), and P-gp (~1000 cpm) phosphorylated by PKC, respectively. In panel F, the relative migration positions of phosphopeptides containing Ser-661 and Ser-667 are indicated. Origins are indicated by arrows. High-voltage electrophoresis was from left to right and chromatography from bottom to top.

[e.g., Boyle et al. (1991)]. The distinct map pattern of PL-S683 and the absence of similar phosphopeptides in the map pattern of PL-5S support the conclusion that Ser-683 is not phosphorylated by PKC in PL-5S.

In order to directly compare the phosphopeptides derived from the fusion proteins with those derived from the native protein, phosphopeptide mapping of P-gp phosphorylated by PKC was performed (Figure 5G). We observed three distinct phosphopeptides, as reported earlier (Chambers et al., 1992), which from sequence analysis (Chambers et al., 1993, 1994) represent phosphorylation of three sites, namely, Ser-667, Ser-671, and Ser-661, in order of migration in the second dimension. No evidence of the phosphopeptides derived from the Ser-683 site was observed. The two most heavily labeled phosphopeptides from P-gp are those containing Ser-667 and Ser-661. Thus, PKC recognizes similar sites in both the native protein and PL-5S.

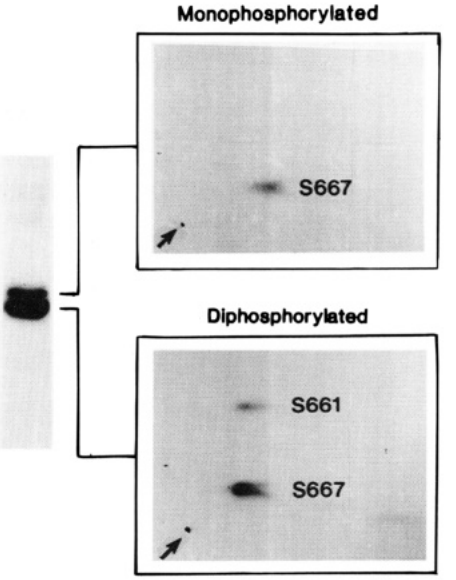

FIGURE 6: Nonuniform phosphorylation of major sites. PL-S661/S667 was phosphorylated by V1 kinase, and two phosphorylated species were resolved by 12% acrylamide SDS−PAGE, as shown on the left. The individual bands were excised and subjected to phosphopeptide mapping analysis, as described in the legend to Figure 5. Upper band = 400 cpm; lower band = 1000 cpm.

Phosphorylation time-courses indicated that under the conditions used both PL-5S and PL-S661/S667 were near (95%) maximally phosphorylated after a 30 min incubation. The stoichiometries of phosphorylation were lower than the maximum expected 2 mol of phosphate/mol of peptide, presumably due to steric hindrance caused by the GST moiety. After shorter incubations or when less active kinase preparations were utilized, we found that PL-S661/S667 upon phosphorylation by the V1 kinase (Figure 6) or by PKC (data not shown) resolved into two phosphorylated species on SDS−PAGE. Phosphopeptide mapping indicated that the upper component of the doublet contained a single phosphopeptide (Ser-667) and the lower component contained two phosphopeptides (Ser-667 and Ser-661). Monophosphorylated PL-S661/S667 containing phosphorylated Ser-661 was not detected. These results suggest the possibility that phosphorylation proceeded in an ordered rather than random fashion with Ser-667 modified first or that phosphorylation of Ser-667 precludes that of Ser-661 in the GST fusion protein.

DISCUSSION

The role of phosphorylation in the mechanism of P-gp-mediated drug transport has been difficult to establish in part because many kinase inhibitors appear to interact with the substrate binding regions of P-gp. Staurosporine (Sato et al., 1990), staurosporine derivatives (Miyamoto et al., 1993), calphostin C (Gupta et al., 1994), and certain isoquinolinesulfonamide (H7) derivatives (Miyamoto et al., 1990) have all been reported to exhibit this property and may therefore increase drug accumulation in MDR cells by competing with drugs for transport distinct from, or in addition to, effects on P-gp phosphorylation. We have begun to explore an alternative approach where specific phosphorylation sites are identified and substituted with nonphosphorylatable amino

Table 1: Summary of PKC- and V1 Kinase-Catalyzed Phosphorylation of GST−PL Fusion Proteins

fusion protein	P-gp sequence	relative phosphorylation by V1 kinase	relative phosphorylation by PKC
GST		−	−
GST−PL-5A	5 Ala	−	−
GST−PL-5S	5 Ser (wild type)	++++	++++
GST−PL-S661	Ser−661, 4 Ala	++	+++
GST−PL-S667	Ser−667, 4 Ala	++	+++
GST−PL-S671	Ser−671, 4 Ala	−	−
GST−PL-S675	Ser−675, 4 Ala	−	−
GST−PL-S683	Ser−683, 4 Ala	−	+
GST−PL-S661/S667	Ser−661/Ser−667, 3 Ala	+++	+++

acids, with the eventual goal of analyzing the properties of cells transfected with phosphorylation mutants of P-gp. Such an approach depends on identification of the actual sites of phosphorylation since, on the basis of consensus sequence motifs, P-gp harbors several dozen potential sites rendering random mutagenesis impractical.

Our previous biochemical studies with human *MDR*1 P-gp have indicated that the major sites of PKC phosphorylation *in vitro* occur in the linker region and include Ser-661, Ser-667, and Ser-671 (Chambers et al., 1993, 1994). On the basis of comparative two-dimensional phosphopeptide mapping (Chambers et al., 1992), these three sites also appear to be the major sites of human P-gp phosphorylation in intact cells. Since these sites are clustered in a single region of the molecule, we were able to express this portion of P-gp as a fusion protein with GST and perform a mutational analysis of the phosphorylation sites. This fusion protein expression system offered significant advantages over the use of synthetic peptides in that a series of mutant proteins with systematic Ser to Ala replacements were readily generated and purified. This methodology facilitated the number of combinations of substitutions that could be made, and also enabled us to determine whether the phosphorylation of one site was dependent or not on the phosphorylation of other sites. The fusion proteins were utilized to identify and confirm the location of the PKC sites and, moreover, to test and compare the specificity of the recently purified V1 kinase. This kinase is likely responsible for the *in vivo* phosphorylation of P-gp in KB-V1 cells[2] and for the major phosphorylation of P-gp in transporting vesicles derived from these cells (Lelong et al., 1994).

The main findings are summarized in Table 1. The V1 kinase preparation exhibited a well-defined specificity, phosphorylating Ser-661 and Ser-667 in PL-5S, recognizing both these sites in PL-S661/S667, and phosphorylating each site independently in PL-S661 and PL-S667. Although phosphorylation of Ser-661 or Ser-667 did not require the presence of the other site, when both sites were present, phosphorylation proceeded in a nonuniform fashion with either Ser-667 modified first or its phosphorylation precluding phosphorylation of Ser-661 (Figure 6). Analysis of the phosphorylation of PL-5S by PKC indicated that only two of the five PKC consensus sites, Ser-661 and Ser-667, were phosphorylated (Figure 5), and these were recognized independently of the other (Figure 4A). In a previous study using a synthetic peptide substrate, Ser-671 as well as Ser-661 and Ser-667 was phosphorylated by PKC (Chambers et al., 1994). Ser-671 is also phosphorylated in native P-gp (Chambers et al., 1993; Figure 5G). The reason for the lack of recognition by PKC of Ser-671 in PL-5S (and PL-S671) is unclear but may be due to steric hindrance caused by the GST moiety. This possibility could be tested by exploiting the thrombin cleavage site present in the pGEX-2T vector and examining phosphorylation of the P-gp peptide released from the GST moiety. However, attempts to isolate such a peptide in sufficient quantity and purity for biochemical studies have thus far been unsuccessful.

Unlike the V1 kinase, PKC also phosphorylated Ser-683, but only in the single-serine PL-S683 construct and not in the context of the wild-type sequence in PL-5S or in native Pgp. Thus Ser-683 appears to represent a nonpreferred PKC site which becomes phosphorylated only when the preferred sites are absent. Mutation of Ser-661 and Ser-667 may cause a conformational change in the peptide, allowing Ser-683 to be recognized by PKC. The phosphorylation of PL-S683 by PKC, but not by the V1 kinase, distinguished the two kinase preparations which otherwise exhibited a very similar specificity. This subtle but distinct difference in substrate specificity suggests that the V1 kinase represents a subclass of, or a single (and perhaps novel) member of, the PKC family of isoforms. Such a conclusion would be consistent with earlier work indicating that the P-gp kinase in KB-V1 cells exhibited PKC-like properties (Chambers et al., 1990, 1992).

In conclusion, the fusion protein system described here has provided a convenient method to identify and confirm the location of the phosphorylation sites in the linker region of the *MDR*1 gene product and to further characterize the properties of a novel V1 kinase preparation. A series of full-length cDNAs encoding well-defined phosphorylation site mutants of P-gp can now be constructed for transfection studies in mammalian cells. The majority of phosphorylated proteins contain multiple sites, and for some of these, for example, glycogen synthase, individual sites are phosphorylated in an ordered or hierarchical manner (Roach, 1991). The strategy employed here would appear to be ideally suited and of general applicability in the examination of multisite and hierarchical protein phosphorylation in other systems.

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