Phosphorylation by protein kinase C and cyclic AMP-dependent protein kinase of synthetic peptides derived from the linker region of human P-glycoprotein

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Specific sites in the linker region of human P-glycoprotein phosphorylated by protein kinase C (PKC) were identified by means of a synthetic peptide substrate, PG-2, corresponding to residues 656–689 from this region of the molecule. As PG-2 has several sequences of the type recognized by the cyclic AMP-dependent protein kinase (PKA), PG-2 was also tested as a substrate for PKA. PG-2 was phosphorylated by purified PKC in a Ca^{2+}/phospholipid-dependent manner, with a K_m of 1.3 μM, and to a maximum stoichiometry of 2.9±0.1 mol of phosphate/mol of peptide. Sequence analysis of tryptic fragments of PG-2 phosphorylated by PKC identified Ser-661, Ser-667 and Ser-671 as the three sites of phosphorylation. PG-2 was also found to be phosphorylated by purified PKA in a cyclic AMP-dependent manner, with a K_m of 21 μM, and to a maximum stoichiometry of 2.6±0.2 mol of phosphate/mol of peptide. Ser-667, Ser-671 and Ser-683 were phosphorylated by PKA. Truncated peptides of PG-2 were utilized to confirm that Ser-661 was PKC-specific and Ser-683 was PKA-specific. Further studies showed that PG-2 acted as a competitive substrate for the P-glycoprotein kinase present in membranes from multidrug-resistant human KB cells. The membrane kinase phosphorylated PG-2 mainly on Ser-661, Ser-667 and Ser-671. These results show that human P-glycoprotein can be phosphorylated by at least two protein kinases, stimulated by different second-messenger systems, which exhibit both overlapping and unique specificities for phosphorylation of multiple sites in the linker region of the molecule.

INTRODUCTION

A major limiting factor in the effectiveness of cancer chemotherapy is acquired or intrinsic multidrug resistance (MDR). Cross-resistance to anticancer drugs such as the vinca alkaloids, anthracyclines and epipodophyllotoxins is due to overexpression of a membrane glycoprotein, P-glycoprotein (Pgp), an energy-dependent drug-efflux pump [1,2]. Sequence and hydropathy analyses predict Pgp to form two homologous halves with each half containing one nucleotide-binding domain and six trans-membrane domains [3,4]. Although modifications to some features of this model have been proposed [5,6], this membrane topology places Pgp in the ATP-binding cassette (ABC) super-family of active transporters [7]. Another member of this class of transporters is the cystic fibrosis transmembrane conductance regulator (CFTR), a cyclic AMP-regulated chloride ion channel [8].

An understanding of Pgp structure, function and regulation is central to defining strategies for Pgp inhibition and reversal of MDR. One potential regulatory mechanism is covalent modification by phosphorylation, a universal feature of Pgps expressed in MDR cell lines (e.g. [9–12]). Our studies with human epi-dermoid carcinoma KB-V1 cells have suggested that protein kinase C (PKC) or a closely related enzyme plays a major role in Pgp phosphorylation [13–15]. However, whereas non-selective kinase inhibitors such as staurosporine completely inhibited Pgp phosphorylation [12,15], PKC-selective inhibitors such as cal-phostin C and an alkylphospholipid, 1-O-octadecyl-2-O-methyl-glycero-3-phosphocholine, were only partially effective [15]. In addition, Pgp can be phosphorylated *in vitro* by protein kinases other than PKC, including the cyclic AMP-dependent protein kinase (PKA) [16] and an incompletely characterized Ca^{2+}-independent phospholipid-dependent protein kinase [17]. Therefore the possibility exists for the phosphorylation of Pgp by more than one kinase activated by different signal-transduction mechanisms.

Studies of drug accumulation in MDR cells in response to agents that increase or decrease Pgp phosphorylation have consistently found an inverse relationship between the amount of drug accumulated and the extent of Pgp phosphorylation [12–15,18–20]. These findings suggest a stimulatory role for phosphorylation in Pgp-mediated drug transport. In order to understand in more depth the role of phosphorylation in Pgp function, it is necessary to identify the number and location in the primary sequence of the phosphorylated residues. Recently, we have shown that PKC phosphorylates Pgp at multiple sites in the linker region between the two halves of the molecule [21]. This phosphorylated region, which also contains consensus PKA sites, may represent a regulatory domain, perhaps analogous to the R-domain of CFTR, a target of multisite regulatory phosphorylation by PKA and PKC (e.g. [22]). We have identified two PKC sites, Ser-661 and Ser-671, in human Pgp [21]; additional sites were not identified owing to difficulties in purifying sufficient amounts of the relevant phosphopeptides. As we plan to assess the function of phosphorylation through site-directed mutagenesis, identification of all the relevant phosphorylation sites is critical. We have therefore synthesized a peptide, termed PG-2, with a sequence corresponding to residues

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656–689 of human *MDR 1* Pgp [4], which encompasses the phosphorylated region. Studies with synthetic peptides offer an attractive complementary approach of particular utility for large hydrophobic proteins like Pgp which are difficult to purify and analyse by traditional biochemical techniques. Phosphorylation of PG-2 by PKC and PKA was examined and compared in terms of kinetics, stoichiometry, phosphopeptide mapping and sequence analysis to identify specific phosphorylation sites. We show that PG-2 is an excellent substrate for both PKC and PKA, and that these kinases phosphorylate two sites in common and one unique site each. Protein kinase-specific phosphorylation of the unique sites was confirmed by examination of truncated peptides as substrates. PG-2 was also used as a probe to characterize the endogenous phosphorylation of Pgp in membranes from KB-V1 cells.

MATERIALS AND METHODS

Materials

[γ-^{32}P]ATP was obtained from ICN Radiochemicals; trifluoro-acetic acid (TFA) was from Pierce Chemical Co.; organic solvents were from Fisher Scientific; sequencing-grade trypsin and all other reagents were from Sigma Chemical Co. Porcine brain PKC [23], bovine brain PKA holoenzyme [24] and bovine heart PKA catalytic subunit [25] were prepared as described in the references cited.

Peptide synthesis

The sequence of the synthetic peptide PG-2 (see Figure 1) was based on the published sequence of residues 656–689 of human *MDR 1* Pgp deduced from the cDNA sequence [4]. Truncated peptides, Pgp-(656–666) and Pgp-(679–687), with the residues indicated in parentheses, were also prepared. The peptides were synthesized by the solid-phase method in an Applied Biosystems (Foster City, CA, U.S.A.) model 430A peptide synthesizer using the *t*-butyloxycarbonyl/benzyl protecting group strategy, as described elsewhere [26]. The peptides were purified by reversed-phase h.p.l.c. (RP-h.p.l.c.) on a C18 silica ODS-300 Aquapore column (1 cm × 10 cm, 20 μm particle size, Applied Biosystems [26]), and their purity and structural integrity were confirmed by amino acid analysis, N-terminal sequence analysis, microbore RP-h.p.l.c. and liquid secondary-ion m.s. The concentration of stock solutions of the peptides was determined ($\pm 5\%$ error) by quantitative amino acid composition analysis.

Peptide phosphorylation

Standard reaction mixtures of 0.2 ml, incubated for 10 min at 30 °C, contained 20 mM Tris/HCl, pH 7.5, 5 mM MgCl$_2$, 20 μM peptide and 50 μM [γ-^{32}P]ATP (500 c.p.m./pmol). Phosphorylation with PKC (100 ng) was carried out in the presence or absence of 0.5 mM CaCl$_2$ and 50 μg/ml phospha-tidylserine (PS), and phosphorylation with PKA (50 ng) was carried out in the presence or absence of 20 μM cyclic AMP. In some experiments, the free catalytic subunit of PKA was used. Reactions were terminated by applying 50 μl portions to P81 phosphocellulose filter discs. The filters were washed with 75 mM phosphoric acid, dried and radioactivity was determined by scintillation counting. Phosphorylation kinetics were determined in the standard reaction mixture with 0.1, 0.3, 1, 3, 10, 30 and 100 μM of peptide. Kinetic parameters were evaluated from double-reciprocal Lineweaver–Burk plots. For comparative purposes, kinetic parameters for phosphorylation of histone H1 (Sigma III-S) and a synthetic peptide substrate derived from

cardiac troponin I, TnI-(134–154) [27], were also determined. For the determination of the stoichiometry of phosphorylation, PG-2 was incubated in the standard reaction mixture with 1 mM [γ-^{32}P]ATP (40 c.p.m./pmol). Duplicate samples of 10 μl were removed at 1 h intervals up to 5 h, applied to P81 filters, washed with 75 mM phosphoric acid, and radioactivity was determined.

Purification of tryptic fragments of phosphorylated PG-2

PG-2 (15 nmol) was phosphorylated by PKC or PKA by incubation (3 h, 30 °C) in standard reaction mixtures (0.25 ml) containing 1 mM [γ-^{32}P]ATP (7 c.p.m./pmol). After the addition of TFA to 0.1%, the peptide was purified by injecting the sample on to an analytical Vydac C18 RP-h.p.l.c. column (4.6 mm × 250 mm) equilibrated in 0.06% TFA/water. The gradient was run from 0 to 80% acetonitrile at an increase of 0.8%/min at a flow rate of 0.5 ml/min. Fractions containing phosphorylated PG-2 were eluted at 18–22% acetonitrile and were pooled, dried, dissolved in 0.2 ml of 0.2 M NH$_4$HCO$_3$, pH 8.0, and digested with 1 μg of trypsin for 24 h at 37 °C. Tryptic fragments were separated by RP-h.p.l.c. as above. Fractions containing phosphorylated fragments were pooled, the volume reduced to 50 μl in a Speed-Vac, and 1–2 nmol of each peptide (based on Cerenkov counting) was subjected to amino acid sequence analysis.

Sequence analysis

Automated Edman degradation of the peptides was performed in an Applied Biosystems model 477A protein sequencer and the phenylthiohydantoin (PTH) derivatives were identified and quantified on-line in an Applied Biosystems model 120A PTH analyser. During sequencing, the phosphoserine residues undergo β-elimination to dehydroalanine residues to a greater extent than the non-phosphorylated serine residues. PTH-dehydroalanine, and the adducts of PTH-dehydroalanine with dithiothreitol, denoted Ser*, are recovered in the chromatograms [28]. (Less than 10% of PTH-dehydroalanine undergoes rehydration to PTH-Ser under the conditions used.) A decreased ratio of Ser/Ser* in a cycle is thus a strong indication of the presence of phosphoserine. PTH-Ser(P) is not recovered at all.

Membrane phosphorylation

Membrane vesicles were prepared from KB-V1 cells as described [21], and samples containing 100 μg of protein were incubated in reaction mixtures of volume 0.1 ml containing 20 mM Tris/HCl, pH 7.5, 5 mM MgCl$_2$, 25 mM sucrose, 1 mM phenylmethane-sulphonyl fluoride, 1 mM ouabain, 1 mM sodium orthovanadate, 1 μM okadaic acid and 0.2 mM [γ-^{32}P]ATP (1300 c.p.m./pmol). Reactions were performed in the presence and absence of 20 μM PG-2. After 30 min at 30 °C, reaction mixtures were divided and one-half was analysed by SDS/PAGE using 6% gels [29] and the other half peptide by PAGE using 16.5% gels [30].

Other methods

Two-dimensional cellulose thin-layer tryptic phosphopeptide mapping was performed as described previously [15]. Tryptic phosphopeptides of PG-2 phosphorylated by PKC or PKA were obtained as described above. Tryptic phosphopeptides of Pgp and PG-2 phosphorylated by the membrane kinase were obtained by excising the protein band from the appropriate gel, and processing the sample as detailed previously [15]. The phospho-peptides were separated in the first dimension by electrophoresis

toward the cathode for 3 h at 450 V in a solvent system of pyridine/acetic acid/water (1:10:89, by vol., pH 3.7). Second-dimension ascending chromatography was performed in a solvent system of butan-1-ol/pyridine/acetic acid/water (50:33:1:40, by vol.). Two-dimensional phosphoamino acid analysis was performed as described [21]. Protein concentration was determined with BSA as a standard as described [31].

RESULTS

Kinetics and stoichiometry of phosphorylation of PG-2 by PKC and PKA

The amino acid sequence of PG-2 is shown in Figure 1. The peptide was found to be an excellent PKC substrate, with a $V_{max.}$ value greater than, and a K_m value similar to, those for histone H1 and TnI-(134–154) (Table 1). Among these PKC substrates, PG-2 was the most effective, as judged by the highest V_{max} to K_m ratio, ranking PG-2 as one of the best peptide substrates of PKC documented to date [32]. PG-2 was also found to be an effective substrate for PKA, with a $V_{max.}$ to K_m ratio about 60 % that of histone H1 (Table 1). Similar results were obtained if the free catalytic subunit was used in place of PKA holoenzyme plus cyclic AMP.

The time course and stoichiometry of phosphorylation of PG-2 by PKC or PKA were examined and compared (Figure 2). Phosphorylation by PKC was complete within 3 h and reached a maximum of 2.9 ± 0.1 mol of phosphate/mol of peptide ($n = 4$). Supplementing the reaction with additional enzyme and ATP at 3 h and incubating for a further 2 h did not increase the phosphorylation stoichiometry (Figure 2). Phosphorylation by PKA followed a similar time course, was complete within 2–3 h, reached a maximum of 2.6 ± 0.2 mol of phosphate/mol of peptide ($n = 3$), and similarly was not further increased with supplementary enzyme and ATP. Thus phosphorylation stoichiometry was not limited by kinase inactivation or ATP depletion. Phosphorylation of PG-2 by PKC or PKA was dependent respectively on the presence of Ca^{2+}/PS or cyclic AMP; in their absence, phosphate incorporation was less than 0.1 mol/mol (Figure 2).

Phosphoamino acid analysis and phosphopeptide mapping

Acid hydrolysis and two-dimensional phosphoamino acid analysis of PG-2 maximally phosphorylated by PKC or PKA indicated that phosphorylation occurred exclusively on serine residues (results not shown). Tryptic phosphopeptide maps of PG-2 phosphorylated by PKC showed the presence of three phosphopeptides (Figure 3, spots 1, 2 and 4). A similar analysis of PG-2

654   689
NDSRSSLIRKRSTRRSVRGSQAQDRKLSTKEALD
     ↑   ↑   ↑   ↑   ↑
    661   667 671 675   683
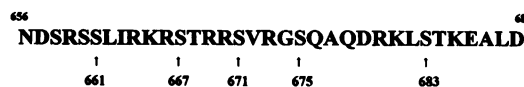
Figure 1   Sequence of PG-2

The amino acid sequence of PG-2 corresponding to residues 656–689 of human Pgp is shown [4]. The residue numbers of the five serines that occur within the PKC/PKA consensus sequences are indicated by arrows.
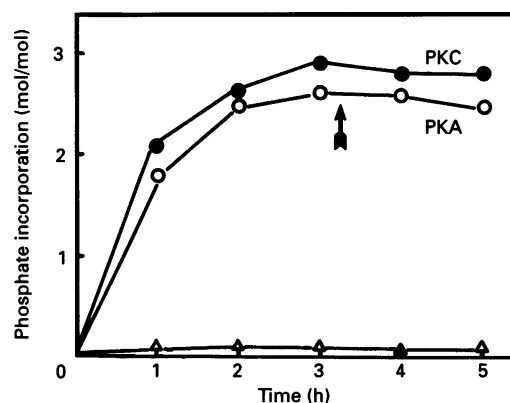
Figure 2   Time course and stoichiometry of phosphorylation of PG-2 by PKC or PKA

PG-2 (4 nmol) was incubated in the standard reaction mixture at 30 °C with [γ-^{32}P]ATP (200 nmol) and either PKC/Ca^{2+}/PS (●) or PKA/cyclic AMP (○). At the times indicated, samples were removed and phosphorylation stoichiometry (mol of phosphate incorporated/mol of PG-2) was determined. The points represent the means of duplicate determinations which differed by less than 3 %, and the data presented are representative of at least three experiments. The arrow indicates the time at which supplementary kinase (50 ng) and ATP (100 nmol) were added to the respective reactions. △, Phosphorylation stoichiometry of less than 0.1 mol/mol observed for PKC in the absence of Ca^{2+}/PS and for PKA in the absence of cyclic AMP.

phosphorylated by PKA also resolved three major phosphopeptides (spots 1, 2 and 3) and one minor phosphopeptide. The map patterns are consistent with the phosphorylation stoichiometry and suggest that each major phosphopeptide contains one phosphorylation site. Phosphorylation of PG-2 by both kinases resulted in the appearance of all four tryptic phosphopeptides. It is evident that phosphopeptides 1 and 2 are common

Table 1   Kinetic parameters for phosphorylation of synthetic peptides and histone H1 by PKC and PKA

TnI-(134–154) is a synthetic peptide, comprising residues 134–154 of bovine cardiac TnI, which contains a major PKC phosphorylation site, Thr-144 [27]. The values presented were estimated from double-reciprocal plots, as described in the Materials and methods section.

| Substrate tested	PKC			PKA		
	$V_{max.}$ (pmol/min)	Apparent K_m (μM)	$V_{max.}/K_m$ ratio	$V_{max.}$ (pmol/min)	Apparent K_m (μM)	$V_{max.}/K_m$ ratio
PG-2	38.5	1.3	29.6	12.3	21	0.58
Histone H1	13.4	0.8	16.7	12.2	13	0.94
TnI-(134–154)	23.0	1.3	17.7	5.1	32	0.16
Pgp-(656–666)	24.1	23.2	1.0	–*	–*	–*
Pgp-(679–687)	–*	–*	–*	13.0	70	0.19

* Not a substrate.
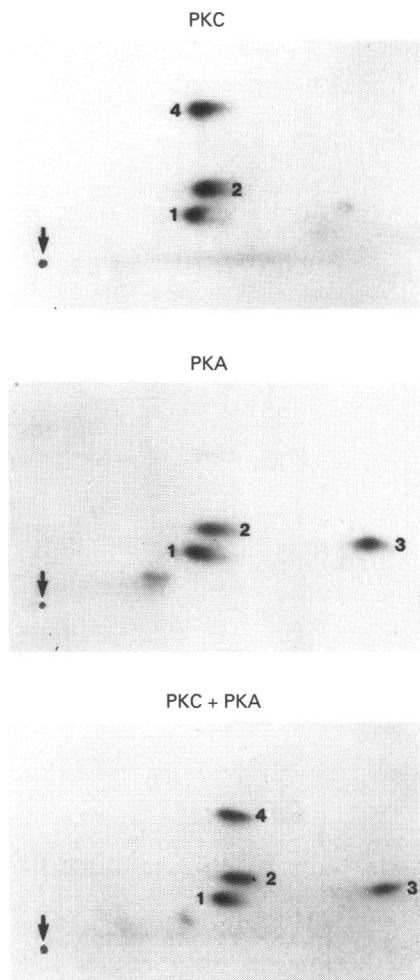
Figure 3 Autoradiographs of two-dimensional phosphopeptide maps of PG-2

PG-2 maximally phosphorylated by PKC, PKA or both was subjected to tryptic phosphopeptide mapping, as described in the Materials and methods section. Peptides were resolved in the first dimension by electrophoresis and in the second dimension by ascending chromatography. Origins are indicated by arrows.

to both kinases, phosphopeptide 4 is PKC-specific, and phosphopeptide 3 is PKA-specific.

Determination of phosphorylation sites

To determine the serine residues phosphorylated, PG-2 was maximally phosphorylated by PKC or PKA, digested with trypsin, and the digests were subjected to RP-h.p.l.c. (Figure 4). The absorbance profile demonstrated an excellent separation of the expected small number of tryptic fragments. In PG-2 phosphorylated by PKC, ^{32}P radioactivity was associated with peptide 1 (unbound), peptide 2 (unbound, retarded) and peptide 4 (eluted at 11–12% acetonitrile). In PG-2 phosphorylated by PKA, ^{32}P radioactivity was associated with peptide 1, peptide 2 and peptide 3 (eluted at 8–9% acetonitrile). The peak numbers correspond to the numbered phosphopeptides of Figure 3, as determined by phosphopeptide maps of the individual peaks (results not shown).
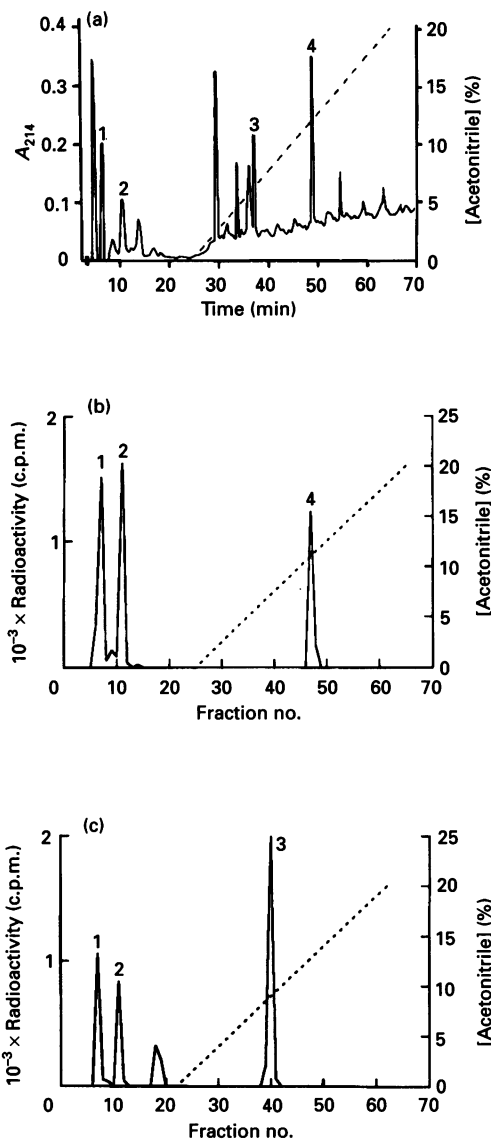
Figure 4 RP-h.p.l.c. of tryptic fragments of phosphorylated PG-2

Tryptic fragments of phosphorylated PG-2 were resolved by chromatography on a C18 column, as described in the Materials and methods section (a). ———, A_{214}; ————, acetonitrile concentration. (b) ^{32}P radioactivity (———) in 0.5 ml fractions after digestion of PKC-phosphorylated PG-2. Results in (a) and (b) are from the same experiment. (c) ^{32}P radioactivity (———) in 0.5 ml of fractions after digestion of PKA-phosphorylated PG-2. The absorbance profile is not shown but was similar to that shown in (a).

The four peaks of Figure 4 were subjected to amino acid sequence analysis (Table 2). Comparison of the sequences obtained with that of PG-2, together with quantification of both Ser and Ser* (derived mainly from phosphoserine, see the Materials and methods section), allowed identification of the phosphorylated residue in each peptide. Thus PKC phosphorylated Ser-661, Ser-667 and Ser-671, and PKA phosphorylated Ser-667, Ser-671 and Ser-683. With the exception of peptide 3, the sequences obtained are those of limit tryptic peptides of PG-2. The presence of a lysine residue at the N-terminus of peptide 3 is presumably due to preferential trypsin cleavage at the adjacent arginine (see Figure 1). The minor PKA phosphopeptide evident in the phosphopeptide map (Figure 3) and h.p.l.c. profile

Table 2 Amino acid sequence analysis of tryptic phosphopeptides of phosphorylated PG-2

The RP-h.p.l.c. peaks 1–4 of Figure 4 (1 nmol of each peptide, based on Cerenkov counting) were subjected to automated Edman degradation and quantification of PTH-derivatives, as described in the Materials and methods section.

Kinase	H.p.l.c. peak	Sequence	Yield (pmol)	Phosphorylation site
PKC	1	S(P)	Ser (27), Ser* (95)	Ser-667
		T	443	
		R	500	
	2	S(P)	Ser (30), Ser* (124)	Ser-671
		V	705	
		R	617	
	4	S	Ser (232), Ser* (112)	
		S(P)	Ser (43), Ser (190)	Ser-661
		L	697	
		I	678	
		R	116	
PKA	1	S(P)	Ser (31), Ser* (71)	Ser-667
		T	595	
		R	210	
	2*	S(P)	Ser (87), Ser* (266)	Ser-671
		V	1584	
		R	257	
	3	K	481	
		L	668	
		S(P)	Ser (38), Ser* (50)	Ser-683
		T	428	
		K	285	

* 2 nmol subjected to sequencing.

(a)

(b)

Figure 5   Inhibition by PG-2 of Pgp phosphorylation in membranes

(a) KB-V1 membrane vesicles (100 μg, containing approx. 20 pmol of Pgp, based on a molecular mass of 150 kDa and assuming Pgp represents 3% of total membrane protein) were incubated under phosphorylation conditions in the absence (−) or presence (+) of 2 nmol of PG-2. Phosphorylation of Pgp (150 kDa) and PG-2 (4 kDa) was visualized respectively by autoradiography after electrophoresis on 8% or 16.5% gels. (b) Autoradiographs of tryptic phosphopeptide maps of Pgp and PG-2 phosphorylated by the membrane-associated kinase. The phosphorylated samples were excised from the gels shown in (a) and processed for trypsin digestion and phosphopeptide mapping, as described in the Materials and methods section.
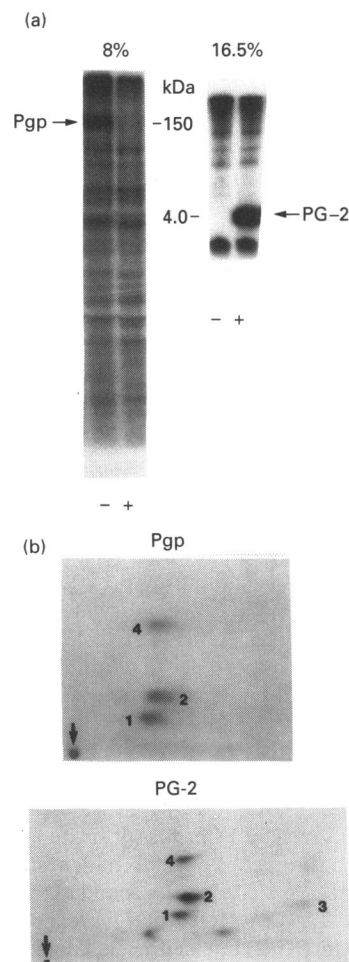
(Figure 4) may represent LSTK, the limit tryptic phosphopeptide of Ser-683.

· These results indicate that PKC and PKA phosphorylated two sites in common and one unique site each. To test further PKC-specific phosphorylation of Ser-661 and PKA-specific phosphorylation of Ser-683, truncated peptides with sequences surrounding each site were prepared. Pgp-(656–666), with the sequence NDSRSS*LIRKR containing Ser-661 (asterisk), was found to be an effective PKC substrate, as reported earlier [21]. However, the peptide was found to be inactive as a substrate for PKA (Table 1). On the other hand, the peptide Pgp-(679–687), with the sequence DRKLSTKEA containing Ser-683, was phosphorylated by PKA but was ineffective as a PKC substrate (Table 1).

PG-2 as a competitive substrate of the Pgp membrane kinase

We have previously shown that when Pgp-containing membrane vesicles from MDR KB-V1 cells were incubated under *in vitro* phosphorylation conditions, Pgp was phosphorylated by endogenous kinase activity [13]. This kinase has not been completely characterized but exhibited the properties of constitutively activated membrane-inserted PKC [13]. It was of interest to determine if the membrane kinase phosphorylated PG-2, and to identify the sites, and therefore membrane phosphorylation reactions were conducted in the absence and presence of the peptide (Figure 5). We found that PG-2 acted as a competitive substrate and, when present in molar excess over Pgp, it completely and specifically inhibited Pgp phosphorylation. Phosphopeptide mapping revealed that incorporation of

phosphate into PG-2 by the endogenous Pgp kinase occurred mainly in phosphopeptides 1, 2 and 4, recognized as containing Ser-667, Ser-671 and Ser-661 respectively. Incorporation of phosphate into phosphopeptide 3, containing the PKA-specific Ser-683 site, occurred at a low level, and other uncharacterized phosphorylated fragments were present. Phosphopeptide mapping of phosphorylated Pgp demonstrated that incorporation of phosphate into Pgp by the membrane kinase occurred in phosphopeptides 1, 2 and 4, i.e. the PKC sites, with no evidence of the PKA-specific site (Figure 5).

DISCUSSION

In this paper we have described the use of a synthetic peptide substrate to analyse Pgp phosphorylation. This approach was undertaken for several reasons. First, Pgp is a large hydrophobic integral membrane protein and as such is difficult to purify and analyse by traditional biochemical techniques. In fact, only recently have satisfactory methods been established for the

purification and reconstitution of Pgp [33]. Secondly, Pgp is highly phoshorylated in intact cells [9–13], and the isolated protein most likely contains significant levels of endogenous phosphate. The presence of endogenous phosphate may in part explain the relatively low stoichiometry of 0.6–1.0 mol of phosphate/mol of Pgp observed for PKC-catalysed *in vitro* phosphorylation [13,21]. Finally, synthetic peptides have a proven record of utility in protein kinase research because of their purity, stability, availability in large amounts, and most importantly because protein kinases recognize the local structure around a phosphorylation site [34,35]. Our synthesis and study of PG-2 has led to the determination of the kinetics, stoichiometry and specific sites of phosphorylation in the linker region of human Pgp catalysed by PKC and PKA. We have found that these protein kinases phosphorylate two sites in common and one unique site each; PKC phosphorylated Ser-661, Ser-667 and Ser-671 and PKA phosphorylated Ser-667, Ser-671 and Ser-683. The sequences surrounding these sites conform well with recognized consensus sequences for these kinases [34,35]. Ser-661 (RSSLIR), Ser-667 (KRSTRR) and Ser-671 (RRSVR) fit the preference of PKC for neighbouring basic residues on both the N- and C-terminal sides of the phosphorylated residue. However, this is true also for Ser-683 (KLSTK), which was not modified by PKC, emphasizing the importance of additional specificity determinants in site recognition. Likewise, the sequences adjacent to the PKA sites Ser-667 (RKRS), Ser-671 (RRS) and Ser-683 (RKLS) conform to the PKA preference for multiple basic residues on the N-terminal side of the phosphorylated residue. Ser-675 (RGS) was not modified by either kinase. The majority of the PKC and PKA sites in various substrate proteins are serine residues, although threonines have been identified in some cases [34]. On the basis of consensus sequence motifs, Thr-668 (KRSTRR) and Thr-684 (RKLSTK) are potential sites for phosphorylation in PG-2, but neither residue was modified by the kinases tested. Experiments with truncated peptides confirmed that Ser-661 was phosphorylated by PKC and not PKA, and the converse was true for Ser-683. These peptides have potential as selective substrates for the two protein kinases.

The electrophoretic and chromatographic properties of the three tryptic phosphopeptides of PG-2 phosphorylated by PKC (Figures 3 and 4) correspond to those of Pgp phosphorylated by PKC documented previously [21]. The PKC sites in Pgp thus appear to be confined to the region of the molecule represented by PG-2. The phosphopeptide maps of Pgp isolated from ^{32}P-labelled KB-V1 cells also showed a similar pattern of three major phosphopeptides [15], and in the present study the same map pattern was observed for Pgp phosphorylated by endogenous membrane kinase activity (Figure 5). These observations provide additional evidence that the major Pgp kinase has the site-specificity characteristics of PKC, and that phosphorylation of this large molecule involves a small cluster of serine residues. We did not observe phosphopeptide 3, the PKA-specific Ser-683 site, in the *in vivo* phosphopeptide maps [15]. Although this site may not be modified in unstimulated KB-V1 cells, it remains to be established whether phosphorylation of Ser-683 occurs in response to agents that activate PKA, e.g. dibutyryl cyclic AMP or forskolin. To our knowledge, the effect of these agents on Pgp phosphorylation in intact MDR cells has not been reported, and thus a physiological role for PKA in Pgp phosphorylation remains unclear. Nonetheless, our studies with PG-2 clearly indicate the presence of specific sites for PKA, and thus the potential exists for phosphorylation of Pgp and modification of function through a cyclic AMP-regulated pathway.

The linker regions of Pgps from different species are, overall, less well conserved than the transmembrane domains and ATP-

binding domains [36]. However, the portion of the linker region corresponding to PG-2 in human Pgp is highly conserved in mouse *mdr 3* [37] and hamster *pgp 1* [38], rodent homologues of human *MDR 1*. Like human Pgp, these rodent Pgps contain multiple PKC/PKA consensus phosphorylation sites in this region. In fact, the sequence around the PKA-specific site Ser-683, QDRKLSTKEALD (Figure 1), is perfectly conserved, and the sequence encompassing the three PKC serines is nearly so. The corresponding region in mouse *mdr 1* is less conserved [3], although at least one PKC site and one PKA site are present which correspond respectively to Ser-667 and Ser-683 in human *MDR 1* (G. Orr and S. B. Horwitz, personal communication). This evolutionary conservation of multiple phosphorylation sites in the linker regions of these drug-transporting Pgp members suggests an important role for this domain in Pgp function. However, it remains to be established to what extent phosphorylation influences Pgp structure and function. Phosphorylation could induce conformational alterations within the Pgp molecule, influence the stability of oligomeric structures, for which evidence exists [39], regulate Pgp ATPase activity, alter the drug-binding affinity, or modify an as yet uncovered property of the transporter. Many of these possibilities can be tested with mutants lacking the phosphorylation sites that have now been identified.

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