Identification of the in Vivo Phosphorylation Sites for Acidic-directed Kinases in Murine mdr1b P-glycoprotein

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P-glycoprotein, the multidrug resistance transporter, is phosphorylated in vivo and the major phosphorylation domain has been identified as the linker region (amino acids 629–686). The linker region is a highly charged segment of the transporter in which the negative and positive amino acid side chains are spatially segregated. Both of these charged domains contain several consensus phosphorylation sites for protein kinases. Three of the consensus phosphorylation sites for basic-directed kinases in murine mdr1b P-glycoprotein are utilized in vivo and have been identified as serines 665, 669, and 681. Mutagenesis of all the consensus basic-directed kinase phosphorylation sites in the linker region of human MDR1 P-glycoprotein did not alter the ability of the mutated transporter to confer the multidrug resistance phenotype in stably transfected cell lines. These studies would suggest that phosphorylation/dephosphorylation within the basic domain of the linker region is not directly involved in regulation of drug transporter activity. We now report that the linker region of mdr1b P-glycoprotein is also phosphorylated in vivo within the acidic domain (amino acids 631–658). These sites have been mapped using casein kinase II, a prototypic acidic-directed kinase, and a recombinant mdr1b linker region peptide (amino acids 621–687). Electrospray mass spectrometry demonstrated that casein kinase II could introduce up to five phosphates into the recombinant peptide. Two-dimensional phosphopeptide mapping indicated that all the phosphates were contained in a tryptic peptide consisting of amino acids 631–658. Phosphopeptide mapping of in vivo labeled P-glycoprotein, isolated from either J7.V1-1, a murine vinblastine-resistant cell line, or HeLa cells stably transfected with mdr1b P-glycoprotein cDNA, revealed that this tryptic peptide was phosphorylated in both proteins.

The acquisition of the multidrug resistance (MDR) phenotype in tumor cell lines is associated with the overexpression of a 170,000-dalton plasma membrane glycoprotein, P-glycoprotein (1–3). P-glycoprotein is encoded by two classes of mdr genes, I and II. In humans there is one member of each class, whereas in mouse there are two members (mdr1a and mdr1b) in class I and one member in class II (3, 4). Gene transfer experiments have shown that only class I genes can confer the MDR phenotype. Sequence analysis of the mdr genes indicates that P-glycoproteins are composed of two homologous halves, each containing a nucleotide binding fold (5, 6). Hydropathy plot analysis predicts that each half-domain has six α-helical transmembrane spanning segments with both the N and C termini having a cytoplasmic orientation. The two homologous halves are connected by an intracellular linker region of approximately 60 amino acids. It is generally believed that the class I P-glycoproteins function as ATP-driven drug efflux pumps with a broad specificity for hydrophobic compounds, thereby reducing intracellular drug levels in resistant cells. It has recently been shown that the class II gene encodes a phosphatidylinositol-specific phospholipase C (7).

It is known that P-glycoprotein is phosphorylated in vivo and also in vitro by several kinases including PKA and PKC (8–13). Nevertheless, a physiological role for phosphorylation in the regulation of the transporter has been difficult to document. We have established that the linker region of murine mdr1b P-glycoprotein is the major site of phosphorylation under in vivo labeling conditions (14). This region of the transporter is characterized by its high content of charged amino acids clustered into negative and positive domains (Fig. 1). Although the linker region is the most divergent segment of all mammalian P-glycoproteins, there are several consensus phosphorylation sites for both acidic and basic-directed kinases, which are totally conserved in human, mouse, and hamster class I P-glycoproteins. This could imply some evolutionary pressure for the maintenance of these sites. Consensus phosphorylation sites for basic-directed kinases in murine mdr1b P-glycoprotein, which are present in murine mdr1a, human MDR1, and hamster P-gp2, include serines 659, 665, 669, and 681 (numbering based on murine mdr1b). We have shown in murine mdr1b P-glycoprotein that serines 665, 669, and 681 are all utilized in vivo (15). Nevertheless, mutagenesis studies of the corresponding sites in the human MDR1 have established that phosphorylation within this basic domain is not directly involved in the regulation of drug transport activity (16, 17).

The linker region of P-glycoprotein also contains a cluster of serine/threonine residues in the acidic segment of this domain (Fig. 1), and many of these sites are also conserved in mammalian class I, but not class II, P-glycoproteins. The goal of this present work was to determine whether these acidic-directed kinase phosphorylation sites in murine mdr1b P-glycoprotein were utilized in vivo.

EXPERIMENTAL PROCEDURES

Cells—The vinblastine-resistant cell line, J7.V1-1, which is ~1000-fold resistant to vinblastine, was isolated by stepwise selection from the drug-sensitive macrophage-like cell line J774.2 and maintained in 1 μM...
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**RESULTS**

Two-dimensional Tryptic Phosphopeptide Mapping of *in Vivo* Labeled mdr1b P-glycoprotein Isolated from Mouse J7.V1-1 Cells and HeLa Cells Stably Transfected with mdr1b P-glycoprotein cDNA—The two-dimensional trypptic phosphopeptide maps of *in vivo* ³²P-labeled mdr1b P-glycoprotein, isolated from either the drug-resistant murine cell-line, J7.V1-1, or HeLa cells stably transfected with mdr1b P-glycoprotein cDNA, are shown in Fig. 2 (panels A and B, respectively). The phosphopeptide maps derived from both proteins were qualitatively similar and contained six well resolved phosphopeptides designated 1–6. Although phosphopeptide 2 was a minor labeled component (7% of the applied radioactivity) in P-glycoprotein isolated from J7.V1-1 cells, it was the major ³²P-labeled phosphopeptide (45%) in the stably transfected HeLa cell line. This is indicative of differential phosphate turnover in these two cell lines. We have previously reported that serine was the only amino acid phosphorylated in P-glycoprotein isolated from J7.V1-1 cells. Likewise, phosphoserine was also the only phosphoamino acid detected in ³²P-labeled P-glycoprotein isolated from the stably transfected HeLa cells (Fig. 3). To establish that the majority of the ³²P incorporated into the P-glycoprotein from the stably transfected HeLa cells was also contained within the linker region, as we had previously determined for J7.V1-1 cells (14, 15), the immunoprecipitated ³²P-labeled protein was separated by SDS-PAGE, electroblotted to nitrocellulose, and treated with CNBr. CNBr cleavage of the mdr1b P-glycoprotein generates a peptide fragment (amino acids 627–682) which contains the majority of the linker region and all of the consensus phosphorylation sites (14). This CNBr fragment, and also the bacterially expressed mdr1b linker region peptide (amino acids 621–688), run anomalously on SDS-PAGE displaying apparent masses approximately twice their predicted masses (14). This aberrant electrophoretic mobility is probably due to the large number of acidic residues in this region of P-glycoprotein. Under the conditions used, CNBr digestion liberated >90% of the radioactivity from the nitrocellulose. As can be seen from Fig. 4, all of the CNBr-released radioactivity was contained in a ~12-kDa peptide which was specifically recognized by an antibody raised against a synthetic peptide corresponding to amino acids 665–682 of the murine mdr1b P-glycoprotein. The linker region, therefore, is the major phosphorylation domain in the mdr1b P-glycoprotein isolated from both the drug-resistant murine and the stably transfected human cell lines. Moreover, the same serines residues within the linker region are undergoing phosphate turnover, albeit at different rates.

Phosphopeptides 3, 5, and 6 (see Fig. 2, panels A and B) have been identified previously as the phosphopeptidylated derivatives of S(665)IYR, S(681)MK, and S(669)VHR, respectively (15) and are located in the basic domain of the mdr1b linker region. These phosphopeptides were identified by combined ESI-MS and two-dimensional peptide mapping of the HPLC-purified tryptic phosphopeptides obtained from the *in vitro* PKA- and PKC-phosphorylated recombinant mdr1b linker peptides. We were unable, however, to establish the identity of phosphopeptide 4, since no identifiable ions for this phosphopeptide were observed by ESI-MS (15). To identify phosphopeptide 4, both Ser-669 and Ser-681 were individually mutated to alanine. The serine to alanine substitutions were introduced into the mdr1b P-glycoprotein cDNA by PCR-based methods and the mutated cDNAs stably expressed in HeLa cells (15). Fig. 2 (panel C) shows the tryptic phosphopeptide map derived from the *in vivo* ³²P-labeled S681A mutated mdr1b P-glycoprotein and it is lacking both phosphopeptides 4 and 5 but contains the other constellation of phosphopeptides, i.e. 1, 2, 3, and 6. In contrast, mutagenesis of Ser-669 to alanine resulted in the disappearance of phosphopeptide 6 but not of phosphopeptides 4 and 5 (Fig. 2, panel D). These studies demonstrate unequivocally that phosphopeptide 4 is a partial digestion product of Ser-669.

Thus phosphopeptides 3–6 are derived from three of the four basic-directed kinase sites (Ser-665, Ser-669, and Ser-681) in...
the linker region of \textit{mdr1b} P-glycoprotein.

The remaining unidentified consensus phosphorylation site for basic-directed kinases in the linker region is Ser-659. Unfortunately neither PKA or PKC phosphorylated Ser-659 \textit{in vitro}, and thus we were unable to determine its position on two-dimensional peptide maps (15). To map this particular tryptic phosphopeptide, it was necessary to chemically synthesize two peptides, ESKSPPLIR and SPPLIR. Reverse phase HPLC analysis of trypsin-treated ESKSPPLIR revealed the presence of a new peptide with a retention time identical to SPPLIR, thereby demonstrating that the presence of the serine phosphate did not inhibit tryptic cleavage of the KS\textsubscript{P} bond. This implies that if Ser-659 was phosphorylated \textit{in vivo}, it would be contained in the tryptic peptide SPLIR. However, the chemically synthesized peptide S\textsubscript{P}PLIR did not migrate with either phosphopeptide 1 or 2. Rather the migration pattern of the peptide was similar to that of phosphopeptide 3, \textit{i.e.} S\textsubscript{P}YR (Fig. 7, panel D). This is not surprising since both S\textsubscript{P}PLIR and S\textsubscript{P}YR possess the same net charge at pH 1.9 and have a similar hydrophobicity index, based on the data of Boyle \textit{et al.} (23). Therefore, Ser-659 is not contained within phosphopeptides 1 or 2.

**Identification of a Second Phosphorylation Domain in the \textit{mdr1b} Linker Region**—Since the location of all the tryptic phosphopeptides derived from consensus phosphorylation sites in the basic region have been established, phosphopeptides 1 and 2 must be derived from some other domain of the linker region. The linker region of class I P-glycoproteins also contains several consensus phosphorylation sites for acidic-directed kinases. We have used CKII, a prototypic acidic-directed
kinase, and a recombinant mdr1b linker peptide to locate the position of tryptic phosphopeptides derived from these sites on two-dimensional peptide maps. The primary consensus phosphorylation motif for CKII is S/TXXD/E (25). In the murine mdr1b linker region this motif occurs four times at Ser-643, Ser-645, Thr-653, and Ser-681 (Fig. 1). In addition, phosphoserine/phosphothreonine can substitute for the aspartate/glutamate residue in the above motif thereby also acting as a determinant for CKII phosphorylation (25). For example, in mdr1b P-glycoprotein, phosphorylation of Thr-653 can potentially act as a determinant for phosphorylation of a secondary site, Ser-650, which in turn can direct phosphorylation of Thr-647 (Fig. 1). All of the primary and secondary CKII phosphorylation sites in mdr1b P-glycoprotein are conserved in human MDR1.

CKII can phosphorylate the bacterially expressed GST-mdr1b linker region peptide (amino acids 621–687) under in vitro labeling conditions. The time course for phosphate incorporation into the GST-linker peptide is shown in Fig. 5. Under the conditions used, maximum levels of phosphate incorporation (4.3 mol of $^{32}$P/mole of protein) were achieved in approximately 6 h. No effort was made to optimize the rate of phosphate incorporation. A similar stoichiometry was observed with the thrombin-released mdr1b linker peptide (data not shown). Recombinant GST was not a substrate for CKII (Fig. 5). Both the maximally phosphorylated and the non-phosphorylated GST-linker peptide were purified by reverse phase HPLC and analyzed by ESI-MS (Fig. 6). The masses of the major ions observed in the phosphorylated GST fusion protein were 317 and 396 daltons, respectively, greater than the mass of the non-phosphorylated protein. Since the addition of each phosphate group increases the mass of a protein by 80 daltons, it is apparent that these phosphorylated species contain four and five phosphate groups, respectively.

Inspection of the deduced amino acid sequence of the mdr1b linker region reveals that complete tryptic digestion will give rise to a peptide, amino acids 631–658, which contains three of the four primary, and both secondary, CKII phosphorylation sites (Fig. 1). The remaining primary CKII site, Ser-681, will be contained in the tryptic peptide LSMK. The two-dimensional tryptic phosphopeptide maps of the in vitro CKII-phosphorylated recombinant mdr1b linker peptide, at different extents of phosphate incorporation (0.2 (5 min) and 3.4 (240 min) mol of $^{32}$P/mole of protein), are shown in Fig. 7 (panels A and B, respectively). Four tryptic phosphopeptides, designated A–D, were found as a well resolved charge train near the origin in the 240-min time point. This time point was chosen simply to illustrate the complete charge train. The orientation of these phosphopeptides, on an upward diagonal toward the anode, indicated that they were the same peptide with differing extents of phosphate incorporation (23). Since phosphopeptides 4 and 5 were previously shown to be derived from Ser-681 and are not observed in these tryptic peptide maps, it appears that CKII does not utilize Ser-681. Although ESI-MS indicated the presence of a penta-phosphorylated peptide only four phosphopeptides were observed on the tryptic phosphopeptide maps. Co-mixing of the 5- and 240-min tryptic digests never revealed the presence of five phosphopeptides (data not shown). Also, the phosphopeptide map of the maximally phosphorylated protein did not contain an additional phosphopeptide to the left of phosphopeptide D in the 240-min digest (data not shown). There are two explanations for our inability to detect five phosphopeptides by peptide mapping. First, and what we believe to be the least likely, is that two of the charged forms are not resolved under the separation conditions employed. The second explanation is that the mono-phosphorylated peptide never existed in free solution. Two of the primary CKII phosphorylation sites (Ser-643 and Ser-645) are in close proximity. It is conceivable that the CKII can phosphorylate both sites without the mono-phosphorylated form dissociating from the enzyme and for this reason we believe that phosphopeptide A in Fig. 7 is a bis-phosphorylated peptide. Efforts to produce a mono-phosphorylated peptide by alkaline phosphatase treatment were unsuccessful.

Fig. 7 (panel C) is the phosphopeptide map of a mixture of the tryptic phosphopeptides derived from both in vivo labeled P-glycoprotein, isolated from the J7.V1-1 cell line (Fig. 2, panel A), and the CK II-phosphorylated recombinant mdr1b linker
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peptide (Fig. 7, panel A, 5-min time point). Phosphopeptide 1 of the in vivo digest co-migrates with phosphopeptide A from the in vitro digest and is thus derived from the acidic domain of the linker region. For the reasons discussed above, we believe that this peptide is a bis-phosphorylated derivative. The remaining unidentified phosphopeptide from the tryptic digest of in vivo $^{32}$P-labeled mdr1b P-glycoprotein is phosphopeptide 2. Since we know (a) that only serine is phosphorylated in vivo, (b) that the linker region is the major phosphorylation domain in mdr1b P-glycoprotein, and (c) the location of all the tryptic phosphopeptides derived from serine residues in the basic domain of the linker region, then phosphopeptide 2 must be derived from the acidic domain peptide (amino acids 631–658). The only remaining potential phosphorylation sites in the mdr1b linker region are two threonine residues at positions 627 and 629 (Fig. 1). The location of phosphopeptide 2 on peptide maps, relative to phosphopeptide 1/A, suggests that it contains a single phosphate. Thus, the major $^{32}$P-labeled tryptic peptide in the mdr1b-transfected HeLa cells is derived from the acidic domain of the linker region.

DISCUSSION

The linker region has been identified as the major phosphorylation domain in mammalian class I P-glycoproteins. This segment of the transporter serves to connect the two homologous halves of the protein and is characterized by its high content of charged amino acids segregated into negatively and positively charged domains. Studies to date have concentrated on the identification of phosphorylation sites for basic-directed kinases within the linker region and the physiological relevance of phosphorylation/dephosphorylation reactions at these residues. We have established that three of the four consensus phosphorylation sites for basic-directed kinases in the murine mdr1b P-glycoprotein (serines 665, 669, and 681) undergo active phosphate turnover in vivo (this study and Ref. 15). However, mutagenesis of all the consensus basic-directed kinase phosphorylation sites within the linker region of human MDR1 P-glycoprotein to a non-phosphorylatable amino acid, either aspartate or alanine, clearly established that phosphorylation of these residues is not involved directly in the regulation of drug transport activity (16, 17). It has not been excluded, however, that phosphorylation within this basic domain does not affect some other aspect of P-glycoprotein function, e.g. specificity, quaternary structure, processing/turover rates, or subcellular localization. Metabolic labeling of cells expressing the mutant human P-glycoprotein, lacking all the potential phosphorylation sites for basic-directed kinases, with $^{32}$P showed that the transporter was poorly phosphorylated in vivo (16). However, two-dimensional tryptic phosphopeptide mapping of the wild type and mutant P-glycoproteins was not performed so the phosphorylation status of the acidic domain of the human MDR1 linker region is not known.

Although metabolic labeling of proteins with $^{32}$P in intact cells is a powerful technique for surveying the phosphorylation status of specific proteins under different experimental conditions, the technique is not without problems (26). Of special concern, especially when used in combination with peptide mapping for phosphorylation site identification, is that the individual phosphorylation sites within a protein are not uniformly labeled. In proteins already synthesized, only those phosphoamino acids which are undergoing turnover will be labeled. However, not all metabolically active phosphorylation sites turnover at the same rate. Furthermore, in the case of metabolically stable phosphorylation sites only those phosphoamino acids derived from proteins synthesized during the pulse period will be labeled. If the half-life of the protein of interest is long, as is the case with P-glycoprotein (27, 28), compared with the pulse period with $^{32}$P, (generally 3–5 h), these sites will be poorly labeled. Therefore, the radioactivity incorporated into specific sites during the pulse labeling period is not a measure of the relative stoichiometry of phosphate incorporation but rather a reflection of the rates of phosphate turnover. This is graphically illustrated in this present study where one specific tryptic phosphopeptide from mdr1b P-glycoprotein (i.e. phosphopeptide 2) is a minor labeled component in the drug-resistant murine J7.V1-1 cell line, but is the major phosphopeptide in the transporter isolated from HeLa cells stably transfected with mdr1b P-glycoprotein cDNA.

All of the in vivo labeled phosphorylation sites in the murine mdr1b P-glycoprotein now have been identified and their positions on two-dimensional tryptic maps located (Fig. 7D). This information will allow the examination of the phosphorylation status of individual serines under a variety of conditions such as the interactions of drugs and reversing agents with P-glycoprotein in cells. Most significantly, our studies have established the acidic domain (amino acids 631–658), in addition to the basic domain (amino acids 659–681), of the murine mdr1b linker region is phosphorylated in vivo. We believe, therefore, that it is premature to exclude at this time the
possible involvement of phosphorylation as a direct modulator of drug transport activity. Within this acidic domain there are five serines, at positions 643, 645, 650, 654, and 657, that could be phosphorylated in vivo. Our ability to assign phosphorylation within the basic domain of the linker region to specific serine residues was due to the fact that tryptic digestion gave rise to unique peptides containing single serine residues (15). The lack of suitable protease sites in the acidic domain precludes a similar analysis in this study. It is also important to stress that although CKII was used in vitro to map the tryptic phosphopeptides containing these residues, the results do not imply that CKII phosphorylates the protein in vivo. Mutagenesis studies will be required to determine unambiguously which of these serines are phosphorylated in vivo and whether this phosphorylation, alone or in combination with phosphorylation within the basic domain of the linker region, is physiologically important to the functioning of the transporter.

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