Synthetic hydrophobic peptides are substrates for P-glycoprotein and stimulate drug transport

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P-Glycoprotein functions as an ATP-driven active efflux pump for many natural products and chemotherapeutic drugs. Hydrophobic peptides have been shown to block drug uptake by P-glycoprotein, indicating that they might be transport substrates. The present study examines the interaction of the synthetic peptide series NAc-L_n-Y-amide with the multidrug transporter. Several peptides in this series caused up to 3.5-fold enhancement of colchicine accumulation in membrane vesicles from multidrug resistant (MDR) cells, which suggests the existence of novel interactions between the binding sites for peptides and drug. Peptides did not stimulate vinblastine transport, which was inhibited as expected for competing substrates. These peptides displayed modest stimulatory effects on the ATPase activity of P-glycoprotein. None blocked azidopine photoaffinity labelling,

showing that they probably occupy a binding site separate from that for the drug. Studies with ¹²⁵I-labelled NAc-LLY-amide showed that it was transported by P-glycoprotein in both membrane vesicles and reconstituted proteoliposomes. Uptake of the peptide was rapid, saturable, osmotically sensitive and occurred against a concentration gradient. The enhancing effect of NAc-LLY-amide on colchicine transport was reciprocated, i.e. colchicine greatly increased the transport of labelled peptide by P-glycoprotein. Peptide transport was also modulated, both positively and negatively, by other MDR spectrum drugs. It is concluded that linear hydrophobic peptides are indeed transported by P-glycoprotein, and some have interactions with drug substrates that result in mutual stimulation of transport.

INTRODUCTION

The resistance of tumours to multiple chemotherapeutic drugs (multidrug resistance, MDR) is a serious barrier to the treatment of human cancer. A major cause of such resistance is the overexpression of a 180 kDa plasma membrane protein, known as P-glycoprotein, which is a member of the ABC (ATP-binding cassette; see [1]), or traffic ATPase [2], superfamily of membrane proteins. P-Glycoprotein is proposed to operate as a drug efflux pump for many structurally unrelated natural products, including colchicine, actinomycin D and chemotherapeutic drugs such as the anthracyclines and *Vinca* alkaloids [3,4]. Functional reconstitution of the protein into proteoliposomes has demonstrated that P-glycoprotein is an active, ATP-dependent transporter, with constitutive ATPase activity that is drug-stimulated [5–7]. Unlike other membrane-bound transporting ATPases, such as the Ca²⁺- and Na⁺K⁺-ATPases, P-glycoprotein ATPase activity does not seem to be tightly coupled to transport activity. The ATPase catalytic site is of low affinity and specificity, and is conformationally flexible [8–10]. P-Glycoprotein is the first ATP-driven membrane transporter to show such unusual characteristics.

The genes encoding P-glycoproteins exist as a small multigene family, with three members in rodents (classes I, II and III) and two in human (classes I and III). The classes I and II P-glycoproteins confer MDR, whereas the class III isoforms do not. The physiological role of the class III P-glycoprotein has recently been established with the demonstration that it is a lipid flippase [11], involved in the export of phosphatidylcholine into the bile from the apical surface of the canalicular cells of the liver [12]. Expression of the classes I and II P-glycoproteins has been

observed in both normal and tumour tissues; however, the physiological substrate(s) for these isoforms are currently unknown.

Since Sharma et al. [13] selected MDR Chinese hamster ovary cells that were resistant to the hydrophobic tripeptide NAc-Leu-Leu-norleucinal (ALLN), several investigators have hypothesized that the multidrug transporter might be capable of transporting peptides. In fact, Raymond et al. [14] have indirectly demonstrated that the mouse *mdr3* protein can transport the yeast a-factor mating pheromone, although the efficiency of this transport remains unclear. We previously showed that the linear peptide gramicidin D, a channel-forming ionophore, is also a substrate for P-glycoprotein [15]. In that study the multidrug transporter was effectively found to interfere with the formation of functional head-to-head gramicidin D dimers in the membrane of intact MDR cells. More recently we have shown that linear peptides such as pepstatin A and leupeptin (NAc-Leu-Leu-arginal) inhibit the transport function of P-glycoprotein in membrane vesicles [16], which suggests that these peptides are substrates for binding and/or transport. Several cyclic ionophores and peptides also interact with P-glycoprotein, on the basis of their ability to inhibit drug transport and stimulate ATPase activity in the same model system [16]. There have been additional reports that hydrophobic peptides [17] and prenyl-cysteine methyl esters [18] increase the ATPase activity of P-glycoprotein in membrane vesicles, suggesting that they interact with the transporter. However, transport of peptides by P-glycoprotein has not been measured directly and remains to be characterized.

The development of chemosensitizers, compounds that block the drug transport capabilities of P-glycoprotein, is obviously a

Abbreviations used: ALLM, NAc-Leu-Leu-methioninal; ALLN, NAc-Leu-Leu-norleucinal; MDR, multidrug resistant/resistance.

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very important goal for the clinical treatment of MDR tumours. Although there have been many studies on the effects of these compounds on both the drug transport and the ATPase activity of P-glycoprotein, their mode of action at the molecular level is unknown, and it is still not clear what features distinguish chemosensitizers from substrates. Toxicity has until now generally been a serious problem for the chemosensitizers tested clinically, which include verapamil, cyclosporin A, quinidine, trifluoperazine and tamoxifen. Hydrophobic peptides might represent a new class of compounds for consideration as potential chemosensitizers of lower toxicity than those currently in use.

Here we demonstrate directly the transport of a synthetic tripeptide by P-glycoprotein, and investigate the structure–activity relationship for the interaction of a series of leucine-rich linear peptides with the transporter. We show that certain peptides greatly increase the transport of colchicine by P-glycoprotein in a model system *in vitro*, and that this effect is reciprocated, i.e. colchicine also enhances the transport of peptide. This phenomenon is not seen, however, with vinblastine and peptides, where each compound is mutually inhibitory to the transport of the other, as would be expected for competing substrates. These results suggest the existence of novel interactions within the P-glycoprotein molecule between the binding/transport site(s) for hydrophobic peptides and certain drugs.

MATERIALS AND METHODS

MDR cell lines and plasma membrane preparation

The MDR Chinese hamster ovary cell lines CH^RC5 and CH^RB30, selected for colchicine resistance [19], were cultured as described previously [20,21]. Plasma membrane vesicles were isolated by a method involving cell disruption by nitrogen cavitation followed by centrifugation on a 35 % (w/w) sucrose cushion [20]. Plasma membrane vesicles were stored at -70°C for no longer than 3 months before use.

Protein determination

Protein was determined by a microplate adaptation of the Bradford assay [22], with BSA as a standard.

Synthetic peptides

ALLN, NAc-Leu-Leu-methioninal (ALLM), leupeptin and LLY (Leu-Leu-Tyr) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). NAc-LLY was synthesized from LLY. Briefly, 3 molar equivalents of acetic anhydride dissolved in dimethylformamide/pyridine (1:5 molar ratio) were added to 5 mg of LLY, and the reaction mixture was stirred at room temperature for 24 h. After drying under a stream of N_2 , the residue was redissolved in $\text{CH}_3\text{CN}/\text{H}_2\text{O}$ (10 %, v/v). The modified peptide was purified by HPLC on a C18 reverse-phase column (Waters DeltaPak), eluting with a continuously increasing gradient of $\text{CH}_3\text{CN}/\text{H}_2\text{O}$ [starting condition 10 % (v/v), flow rate 1 ml/min]. The purity of the final product exceeded 95 %, as determined by HPLC and mass spectrometry. The peptide series NAc-L_n-Y-amide was custom synthesized by Quality Controlled Biochemicals (Hopkinton, MA, U.S.A.). Peptide purity exceeded 98 % as assessed by mass spectrometry and HPLC analysis.

Colchicine and vinblastine transport by P-glycoprotein

Steady-state uptake of [³H]colchicine or [³H]vinblastine into CH^RC5 plasma membrane vesicles was determined with a protocol previously developed in our laboratory [16,23]. Briefly,

membrane vesicles (30 μg of protein) were mixed in a 100 μl final volume of buffer with 1 μM [³H]colchicine or 56 nM [³H]vinblastine, 5 mM Mg^{2+} and 1 mM ATP, together with a regenerating system. After various times at 23°C , vesicles were harvested by rapid filtration on Whatman GF/F filters, and immediately washed with 5 ml of ice-cold buffer. Filters were dried and radioactivity was quantified by liquid-scintillation counting. Peptides were added as stock solutions in DMSO, and controls contained the appropriate levels of solvent, which never exceeded 0.2 % (v/v). This DMSO concentration had no inhibitory effect on ATP-dependent colchicine transport. Drug binding to filters was determined in the absence of membrane vesicles, and non-specific uptake into vesicles was determined in the absence of ATP and a regenerating system.

Median effect analysis

The median effect equation [24,25] describes the relationship between any concentration of a compound and its effect on the system being studied. In this case the inhibition of [³H]colchicine transport into CH^RC5 plasma membrane vesicles was measured at various concentrations of the test peptides. One transformation of the median effect equation is:

$$\log(f_a/f_u) = m \log D - m \log D_m$$

where f_a is the fraction of the system that is affected at a concentration D (in this case the fractional inhibition of equilibrium colchicine uptake), f_u is the fraction of the system that is unaffected at concentration D , D_m is the compound concentration causing 50 % inhibition, and m is a parameter indicating the sigmoidal nature of the dose–effect curve. As previously described [16], a plot of $\log(f_a/f_u)$ against $\log D$ produces a straight line with slope m , and an x -intercept of $\log D_m$.

Measurement of P-glycoprotein ATPase activity

The ATPase activity of P-glycoprotein in CH^RC5 plasma membrane vesicles was determined as described previously [26,27] by measuring the release of inorganic phosphate from ATP. Samples contained CH^RC5 plasma membrane (1–2 μg of protein) with 2 mM ATP and 5 mM Mg^{2+} , which gave maximal ATPase activity. The assay buffer did not contain either Na^+ or K^+ , to avoid contributions to activity from the Na^+K^+ -ATPase; addition of 1 mM ouabain to the assay did not affect the ATPase activity. Membrane vesicles were preincubated with peptides for 5 min before the addition of ATP. Peptides were prepared as stock solutions in DMSO, and controls contained the appropriate DMSO concentration, which never exceeded 0.2 % (v/v).

[³H]Azidopine photoaffinity labelling

Photoaffinity labelling of P-glycoprotein in CH^RC5 membrane vesicles with [³H]azidopine (200 nM, 52 Ci/mmol; Amersham) was performed as described [15,16,20,28], in the presence of various concentrations of peptides. Membrane proteins were analysed by SDS/PAGE on a 7.5 % (w/v) gel, followed by fluorography.

Labelling of NAc-LLY-amide with ¹²⁵I

The synthetic peptide NAc-LLY-amide was labelled with ¹²⁵I on the tyrosine residue by using Iodobeads (Pierce, Rockford, IL, U.S.A.), in accordance with the manufacturer's instructions. Briefly, Na^{125}I (500 μCi ; ICN Pharmaceuticals, Montreal, QC, Canada) was incubated with two beads for 5 min at room temperature in 0.1 M sodium phosphate, pH 7.0. Peptide [600 μg in 10 % (v/v) DMSO] was added to give a final DMSO

concentration of 0.6 % (v/v), and the mixture was vortex-mixed occasionally for 15 min. The radiolabelled peptide was subsequently separated from free iodide as previously described [29]. The reaction mixture was transferred to a 5 ml syringe connected to a C18 SepPak cartridge (Waters, Mississauga, ON, Canada). The cartridge was washed with 20 ml of 0.1 % (v/v) trifluoroacetic acid to remove free iodide, and peptide was then eluted with 4 ml of 0.1 % trifluoroacetic acid/50 % (v/v) acetonitrile, followed by 4 ml of 0.085 % trifluoroacetic acid/acetonitrile. Fractions (1 ml) were collected, and those containing labelled peptide were pooled and evaporated in a SpeedVac Concentrator (Savant), to give a final product with a specific radioactivity in the range $(1.3\text{--}1.7) \times 10^7$ d.p.m./ μmol .

Reconstituted proteoliposomes

P-Glycoprotein was partially purified from CH^RB30 plasma membrane by using the zwitterionic detergent CHAPS, and reconstituted into proteoliposomes of 1:1 (w/w) egg phosphatidylcholine/dipalmitoylphosphatidylethanolamine as previously described [5]. The final proteoliposome lipid-to-protein ratio was approx. 45:1 (w/w).

Peptide transport by P-glycoprotein

Transport of ¹²⁵I-labelled NAc-LLY-amide into CH^RC5 and CH^RB30 plasma membrane vesicles and reconstituted proteoliposomes was performed by a modification of the method used for colchicine transport [5,16,23]. To decrease background binding, filters were presoaked in 1 % (w/v) BSA/50 μM LLY in transport buffer overnight at 4 °C before use. The CH^RB30 cell line has greater P-glycoprotein expression than CH^RC5, and gave approx. 2-fold higher values of ATP-dependent peptide uptake relative to the background, compared with vesicles from CH^RC5. Recovery of CH^RB30 vesicles on the filters was approx. 50 %, identical with that for CH^RC5 vesicles.

RESULTS

Inhibitory effect of hydrophobic tripeptides on colchicine transport by P-glycoprotein

Two series of peptides were tested for their ability to alter the steady-state uptake of [³H]colchicine into CH^RC5 plasma membrane vesicles. The following tripeptides were initially employed:

Table 1 Inhibition of P-glycoprotein-mediated drug transport by hydrophobic tripeptides

Transport of [³H]colchicine into CH^RC5 plasma membrane vesicles was determined in the absence or presence of increasing concentrations of various tripeptides. D_m values were calculated with the median effect analysis (see the Materials and methods section), and represent the concentration of peptide causing 50% inhibition of drug uptake (see [16] for details).

Tripeptide species	C-terminal residue	Overall charge	D_m (μM)
Leupeptin	Argininal	+	80
ALLN	Norleucinal	0	130
ALLM	Methioninal	0	130
LLY	Tyrosine	±	> 350*
NAc-LLY	Tyrosine	—	No inhibition
NAc-LLY-amide	Tyrosine amide	0	Stimulatory

* This concentration was close to the solubility limit of the peptide.

Figure 1 Effect of linear hydrophobic peptides on P-glycoprotein-mediated colchicine transport

The steady-state uptake of 1 μM [³H]colchicine into CH^RC5 plasma membrane vesicles was measured in the presence of 1 mM ATP and a regenerating system, together with increasing concentrations of LLY (Δ), NAc-LLY ($\diamond$), NAc-LLY-amide ($\bullet$), NAc-L₃Y-amide ($\blacklozenge$), NAc-L₄Y-amide ($\blacktriangle$) and NAc-L₅Y-amide ($\blacktriangledown$). Results are presented as a percentage of control ATP-dependent [³H]colchicine uptake relative to membrane vesicles in the absence of peptides (means $\pm$ S.E.M., $n = 3$).

ALLN, ALLM, leupeptin, LLY, NAc-LLY and NAc-LLY-amide. This series allowed an examination of the effects of charge, terminal modifications and C-terminal amino acid identity on the interaction of tripeptide species with P-glycoprotein. Colchicine transport inhibition results were analysed by the median effect analysis to produce the parameter D_m , which is a measure of the peptide concentration required to block drug transport by 50 % (see Table 1). Leupeptin, which has an overall positive charge due to the presence of an arginine residue, was the best inhibitor, with a D_m of 80 μM , whereas the neutral peptides ALLM and ALLN were less effective and were similar to each other. The zwitterionic peptide LLY, which has both termini charged, was a weak inhibitor of drug transport. Acetylation of the N-terminus of LLY to produce a peptide with an overall negative charge (at the C-terminus) abolished the ability of the peptide to inhibit P-glycoprotein transport activity. These observations clearly indicate that P-glycoprotein favours interactions with positively charged or neutral tripeptides over zwitterionic and negatively charged species. The neutral peptide NAc-LLY-amide showed highly unusual behaviour in that, rather than acting as an inhibitor, it increased colchicine uptake by 3.5-fold (see below).

Enhancement of colchicine transport by hydrophobic peptides

The second series of peptides employed in this study was designed to examine the effect of peptide length while preserving hydrophobic nature and overall neutral charge. A tyrosine residue was included at the C-terminus of each peptide to allow the direct measurement of ¹²⁵I-labelled peptide transport (see below). The effects of the peptide series NAc-L_{*n*}Y-amide on uptake of colchicine into CH^RC5 plasma membrane vesicles were unusual in that, rather than acting as competitive inhibitors of colchicine transport, several of these peptides greatly enhanced drug uptake (Figure 1). The tripeptide NAc-LLY-amide resulted in a large increase in ATP-dependent colchicine uptake, which reached more than 3.5-fold at 320 μM . This effect was also seen for the next peptide in the series, NAc-L₃Y-amide, although to a smaller extent, with colchicine uptake increased up to 2.2-fold. As the

Figure 2 Effect of linear hydrophobic peptides on P-glycoprotein-mediated vinblastine transport

The steady-state uptake of 56 nM [^3H]vinblastine into plasma membrane vesicles was measured in the presence of 1 mM ATP and a regenerating system, together with increasing concentrations of LLY (Δ), NAc-LLY-amide ($\bullet$), NAc-L₃Y-amide ($\blacklozenge$), NAc-L₄Y-amide ($\blacktriangle$) and NAc-L₅Y-amide ($\blacktriangledown$). Results are presented as a percentage of control ATP-dependent [^3H]vinblastine uptake relative to membrane vesicles in the absence of peptides (means $\pm$ S.E.M., $n = 3$).

Figure 3 Stimulation of P-glycoprotein ATPase activity in plasma membrane by linear hydrophobic peptides

CH $^{\text{R}}$ C5 plasma membrane vesicles (1.5–2.0 μg of protein) were assayed for Mg^{2+} -ATPase activity in the presence of increasing concentrations of the peptides LLY (Δ), NAc-LLY ($\diamond$), NAc-LLY-amide ($\bullet$), NAc-L₃Y-amide ($\blacklozenge$), NAc-L₄Y-amide ($\blacktriangle$) and NAc-L₅Y-amide ($\blacktriangledown$). Results are presented as a percentage of control Mg^{2+} -dependent ATPase activity measured in the absence of peptides (means $\pm$ S.E.M., $n = 3$).

length of the peptide increased, stimulation of drug uptake declined. The pentapeptide NAc-L₄Y-amide enhanced drug uptake by only 60 %, and the hexapeptide NAc-L₅Y-amide had little effect on drug transport. This is the first report that certain compounds are able to interact with P-glycoprotein to increase drug transport. These results suggest that the stimulatory hydrophobic peptides interact with P-glycoprotein at a specific binding site that is positively linked to the binding/transport site for colchicine.

In contrast, NAc-L_{*n*}Y-amide peptides had only small effects on vinblastine uptake into plasma membrane vesicles (Figure 2), ranging from weak inhibition for the tri- and tetra-peptides to essentially no effect for the penta- and hexa-peptides. LLY was also a weak inhibitor of vinblastine transport, with a D_m of more

than 300 μM . These results indicate that the peptide interaction site on P-glycoprotein is not linked to the vinblastine transport site. In this case, peptides seem to act simply as competing transport substrates (see below for evidence that they are in fact transported by P-glycoprotein). The fact that they are unable to compete well with vinblastine is a reflection of their lower affinity for the transporter (as shown by their D_m values; Table 1), when compared with vinblastine ($D_m = 0.55 \mu\text{M}$).

Stimulation of P-glycoprotein ATPase activity by hydrophobic peptides

It was previously reported that the activity of P-glycoprotein ATPase is increased up to 2.5-fold by several peptides and ionophores [16,17]. Peptides in the series NAc-L_{*n*}Y-amide also increased P-glycoprotein ATPase activity in CH $^{\text{R}}$ C5 plasma membrane, although the highest activation observed did not exceed 40 % (Figure 3). A stimulation by 10–15 % was seen for LLY and NAc-LLY. The extent of catalytic activation increased with peptide length, from 23 % for NAc-LLY-amide to 31 %, 35 % and 39 % for the tetra-, penta- and hexa-peptides respectively. Most of the peptides showed a slight decrease in ATPase stimulation at higher concentrations (tri- and penta-peptides, see Figure 3; result not shown for the hexapeptide). Half-maximal activation of catalytic activity was noted at approx. 50 μM for LLY and NAc-LLY, and at approx. 30 μM for NAc-LLY-amide and NAc-L₃Y-amide. The penta- and hexa-peptides gave half-maximal ATPase stimulation at much lower concentrations, 2 and 6 μM respectively. There does not therefore seem to be a simple correlation between the ATPase stimulation profile of a particular peptide, and the ability of that peptide to increase colchicine transport.

Previous work in our laboratory has shown that linear peptides such as pepstatin A were unable to reverse verapamil activation of P-glycoprotein ATPase activity, whereas the cyclic peptide cyclosporin A, and other drugs/chemosensitizers, were able to do so at relatively low concentrations [16]. None of the peptides in the series NAc-L_{*n*}Y-amide was able to abrogate verapamil stimulation of P-glycoprotein ATPase activity (results not shown), even at concentrations at which they clearly affected both drug transport and ATPase activity. These findings reinforce the idea that linear hydrophobic peptides interact with P-glycoprotein at a site distinct from that for verapamil.

Effect of peptides on azidopine photoaffinity labelling of P-glycoprotein

The ability of a compound to inhibit photoaffinity labelling of P-glycoprotein by the drug substrate azidopine has often been used as an indicator that it competes for a common binding site on the multidrug transporter. However, it seems that not all P-glycoprotein substrates show this behaviour. We previously reported that cyclic peptides (cyclosporin A and valinomycin) inhibited azidopine labelling; however, pepstatin A and leupeptin were unable to do so [16]. None of the peptides in the series NAc-L_{*n*}Y-amide blocked azidopine photolabelling (Figure 4), even at concentrations at which they stimulated P-glycoprotein ATPase and transport activity. These results indicate that the binding site(s) of the peptides used in the present study also do not seem to overlap with the azidopine site.

Transport of ^{125}I -labelled NAc-LLY-amide by P-glycoprotein in plasma membrane vesicles and proteoliposomes

The ability of peptides in the series NAc-L_{*n*}Y-amide to modulate both the drug transport and ATPase activities of P-glycoprotein

Figure 4 Effect of linear hydrophobic peptides on photoaffinity labelling of P-glycoprotein (Pgp) by [^3H]azidopine

CH^RC5 plasma membrane vesicles (100 μg of protein) were incubated with [^3H]azidopine (250 μCi ; 200 nM) in the presence of increasing concentrations of (a) LLY, (b) NAc-LLY, (c) NAc-LLY-amide, (d) NAc-L₃Y-amide, (e) NAc-L₄Y-amide, (f) NAc-L₅Y-amide and (g) valinomycin. After 1 h at room temperature the samples were subjected to UV irradiation for 30 min. After electrophoretic separation, the intensity of P-glycoprotein photolabelling was detected by fluorography; the only visible band was P-glycoprotein, with a molecular mass of 170–180 kDa. Peptide concentrations in μM for each lane are indicated along the bottom of the panels.

suggests that they are likely to be transport substrates themselves. We were able to test this hypothesis directly by radioiodinating NAc-LLY-amide, the peptide demonstrating the highest stimulatory effect on colchicine uptake. Transport measurements were performed in plasma membrane vesicles from the highly drug-resistant Chinese hamster ovary cell lines CH^RB30 and CH^RC5, and the drug-sensitive parent cell line AuxB1. As shown in Figure 5, ATP-dependent uptake of ^{125}I -labelled NAc-LLY-amide into CH^RB30 and CH^RC5 membrane vesicles at a peptide concentration of 50 μM occurred rapidly, reaching a steady state at 5–10 min and remaining stable for up to 30 min. This time course of accumulation of peptide is similar to that noted for colchicine and rhodamine 123 in the same vesicle model system (X. Yu, R. Liu and F. J. Sharom, unpublished work). In both cases only background levels of peptide uptake were noted in the absence of ATP. Maximal accumulation of ^{125}I -labelled NAc-LLY-amide into CH^RB30 vesicles was approximately twice that noted for vesicles from CH^RC5 cells. The former cell line is more highly drug-resistant, and has higher expression levels of P-glycoprotein. In contrast, membrane vesicles from the drug-sensitive AuxB1 cell line showed background levels of peptide uptake. Thus a higher peptide accumulation correlates well with levels of P-glycoprotein expression in this series of MDR cell lines, excluding the possibility of a non-P-glycoprotein-mediated peptide uptake system. Peptide uptake into CH^RB30 vesicles was osmotically sensitive and decreased as the external sucrose concentration increased (results not shown). Uptake of ^{125}I -labelled NAc-LLY-amide was also concentration-dependent and

Figure 5 Time course of ^{125}I -labelled NAc-LLY-amide uptake by plasma membrane vesicles

Peptide uptake at a concentration of 50 μM into plasma membrane vesicles of the MDR cell lines CH^RB30 (●, ○) and CH^RC5 (◆, ◇), and the drug-sensitive parent AuxB1 (▲, △), was determined in the presence (closed symbols) or absence (open symbols) of 1 mM ATP and a regenerating system. Data are presented as nmol of peptide taken up per mg of membrane protein, corrected for background binding to filters, and represent means $\pm$ S.E.M. ($n = 4$).

saturable, reaching a maximal accumulation of 90 nmol/mg of protein at 200 μM peptide (Figure 6). Half-maximal uptake of peptide was observed at a concentration of approx. 100 μM . These results indicate that NAc-LLY-amide is transported by P-glycoprotein in a manner that is both ATP-dependent and saturable.

Partially purified P-glycoprotein (the S_2 fraction) was isolated from CH^RB30 plasma membrane and reconstituted into proteoliposomes of egg phosphatidylcholine/dipalmitoylphosphatidylethanolamine by a method involving rapid removal of detergent by gel filtration [5]. ATP-dependent uptake of ^{125}I -labelled NAc-LLY-amide into these reconstituted proteoliposomes was also observed (Figure 7). A steady-state ATP-dependent peptide uptake of approx. 250 nmol/mg of protein was achieved at about 10 min at 50 μM peptide. The peptide concentration

Figure 6 Dependence of peptide uptake on concentration

The steady-state uptake of ^{125}I -labelled NAc-LLY-amide into CH^RB30 plasma membrane vesicles was measured after 5 min at various peptide concentrations, in the presence (●) or absence (◆) of 1 mM ATP and a regenerating system (RS). Results are presented as nmol of peptide taken up per mg of protein, and represent means $\pm$ S.E.M. ($n = 4$).

Figure 7 Time course of ^{125}I -labelled NAc-LLY-amide uptake by reconstituted proteoliposomes containing P-glycoprotein

^{125}I -labelled NAc-LLY-amide uptake was determined for proteoliposomes reconstituted with partially purified P-glycoprotein from $\text{CH}^{\text{R}}\text{B30}$ cells, in the presence (●) or absence (◆) of 1 mM ATP and a regenerating system (RS). Liposomes of lipid alone showed background levels of peptide uptake, which did not change with time. Peptide concentration was $50\text{ }\mu\text{M}$. Data are presented as nmol of peptide taken up per mg of liposomally incorporated protein, and represent means $\pm$ S.E.M. ($n = 4$).

associated with the proteoliposomes was calculated (see [5] for details). For the purposes of this calculation, it was assumed that all vesicle-associated peptide was intravesicular, which will clearly not be true for a hydrophobic species that partitions into the lipid bilayer. This effect can be corrected for by comparing the apparent intravesicular concentrations in the presence and absence of ATP. The luminal peptide concentration in the absence of ATP (when peptide can enter the liposomes by diffusion only) was substantially higher (1.2 mM) than that external to the proteoliposomes ($50\text{ }\mu\text{M}$). Thus partitioning the peptide into proteoliposomes generated an apparent 25-fold concentration gradient. Similar partitioning effects were previously noted for transport of the hydrophobic drug vinblastine into plasma membrane vesicles [23]. However, in the presence of ATP the peptide concentration in the proteoliposomes increased to 6.4 mM, indicating the generation of a 5.2-fold concentration gradient. Previous experiments showed that proteoliposomes generated a 5.6-fold gradient of the drug colchicine [5]. These results confirm that NAc-LLY-amide is indeed a substrate for transport by P-glycoprotein, and that a substantial peptide concentration gradient is generated. This is the first direct demonstration of active peptide transport by P-glycoprotein.

Modulation of peptide transport by drugs and chemosensitizers

We previously showed that colchicine transport into plasma membrane vesicles [5] and reconstituted proteoliposomes [23] is inhibited in a concentration-dependent manner by MDR-spectrum drugs and chemosensitizers, and also by a variety of hydrophobic natural peptides and ionophores [16], including pepstatin A, gramicidin D, nonactin, valinomycin and cyclosporin A. Because several of the synthetic peptides used in this study did not inhibit colchicine uptake, but rather increased it markedly, it was of interest to determine whether the converse was also true; i.e. whether colchicine increased the transport of peptide. As shown in Figure 8, other MDR drugs and chemosensitizers fell into three distinct groups, depending on their effect on ATP-dependent transport of ^{125}I -labelled NAc-LLY-amide into plasma membrane vesicles. Methotrexate, a drug that

Figure 8 Modulation by drugs and chemosensitizers of P-glycoprotein-mediated transport of ^{125}I -labelled NAc-LLY-amide

The steady-state uptake of ^{125}I -labelled NAc-LLY-amide into $\text{CH}^{\text{R}}\text{B30}$ plasma membrane vesicles at 10 min was measured in the presence of 1 mM ATP and a regenerating system, together with increasing concentrations of (A) colchicine (CLC, ◆), verapamil (VRP, ▼) and cyclosporin A (CYA, ●), and (B) vinblastine (VBL, ▲) and methotrexate (MTX, ■). The concentration of peptide was $50\text{ }\mu\text{M}$. Data points are expressed as percentages of control values measured in the absence of drugs, and represent means $\pm$ S.E.M. ($n = 4$).

is not part of the MDR spectrum, had no effect on peptide transport, as expected (Figure 8B). Vinblastine, a high-affinity substrate for P-glycoprotein, inhibited peptide transport, with half-maximal inhibition occurring at a drug concentration of approx. $2\text{ }\mu\text{M}$ (Figure 8B). This vinblastine concentration is comparable to that required for half-maximal inhibition of P-glycoprotein-mediated transport of colchicine into plasma membrane vesicles and reconstituted proteoliposomes ($1\text{--}2\text{ }\mu\text{M}$) [16,23]. Several other drugs and chemosensitizers increased peptide transport when present in certain concentration ranges (Figure 8A). For the chemosensitizers cyclosporin A and verapamil, we observed maximal stimulation of peptide uptake of 34% and 40% respectively, at approx. $0.1\text{ }\mu\text{M}$. At higher concentrations (more than $1\text{ }\mu\text{M}$), cyclosporin A inhibited peptide transport, whereas verapamil had little effect. Peptide transport was also stimulated by up to 60% by colchicine, with maximal stimulation achieved at approx. $1\text{ }\mu\text{M}$. Higher colchicine concentrations inhibited peptide uptake. These findings suggest that the binding site for the MDR substrate colchicine, but not that for vinblastine, is coupled in some way to the peptide site in a reciprocal fashion. The chemosensitizers cyclosporin A and verapamil also seem to have binding sites that are linked to the peptide transport site in a positive fashion. The existence of such up-regulatory effects on transport by MDR substrates and chemosensitizers has not previously been reported.

DISCUSSION

We and others have shown that several naturally occurring peptides and ionophores both block P-glycoprotein-mediated drug transport [16] and stimulate ATPase activity [16,17]. Here,

a study of the structure-activity relationship for tripeptides has allowed us to determine the effects of overall charge and the identity of the C-terminal amino acid. P-Glycoprotein seems to prefer substrates that contain positively charged N atoms [30], which is consistent with the fact that leupeptin, a tripeptide with a positively charged Arg residue at the C-terminal position, is a good inhibitor of drug transport. Neutral peptides with methionine or norleucine residues at the C-terminal position are less favourable. A peptide carrying an overall negative charge was no longer a transport inhibitor. The effect of a tyrosine residue at the C-terminal position of the tripeptide varies depending on the context in which it is placed (see Table 1). If we consider the uncharged peptides, the substitution of a tyrosine residue for methionine or norleucine at position 3 in NAc-LLX-amide changed its effect on colchicine transport from inhibitory to stimulatory.

We have shown that certain peptides in the series NAc-L_nY-amide can interact with P-glycoprotein to increase drug uptake. One possible explanation for this effect is the existence of a positive allosteric interaction between the binding sites for linear hydrophobic peptides and colchicine. Negative allosteric interactions have already been shown for P-glycoprotein. Ferry et al. [31] have recently demonstrated that the P-glycoprotein binding site for certain dihydropyridines is negatively allosterically coupled to a separate vinblastine-binding site. The effect of the peptide series NAc-L_nY-amide on colchicine transport by P-glycoprotein seems to be correlated with the length of the peptide. The stimulatory effect was maximal for the tripeptide and decreased monotonically with peptide length, so that the hexapeptide had little effect on drug transport. There is some previous indirect evidence for the existence of positive interactions between distinct or overlapping binding sites on P-glycoprotein. Safa et al. [32] demonstrated that prenylamine, a calcium channel blocker, stimulated the azidopine photolabelling of P-glycoprotein. More recently, they reported that colchicine enhanced the binding to P-glycoprotein of tamoxifen aziridine, a novel chemical affinity probe [33].

One important corollary of the proposal that certain peptides enhance drug transport via a positive interaction is that both peptide and drug must interact simultaneously with P-glycoprotein, but at different sites. We have already presented several lines of evidence to support the idea that certain natural linear peptides occupy binding sites that are not linked to, or do not overlap with, the sites for verapamil or azidopine [16]. In contrast, cyclic peptides and ionophores (such as cyclosporin A and valinomycin) seemed to occupy sites that either overlap with, or are linked to, the sites for verapamil and azidopine. This view is confirmed by the present results, which provide strong evidence that the binding/transport site for colchicine is also separate from the site of interaction of the synthetic peptides used in this study.

Interestingly, the enhancing effect of the tripeptide NAc-LLY-amide on colchicine transport was reciprocated, i.e. colchicine greatly increased the uptake of ¹²⁵I-labelled peptide by P-glycoprotein. In addition, the chemosensitizers verapamil and cyclosporin A also acted to stimulate peptide transport, which suggests that they too are able to bind to the multidrug transporter concurrently with peptide, at a separate site. It is not known whether the tripeptide also increases the transport of verapamil and cyclosporin A, but it seems possible in view of the results presented here. Only certain drugs showed this characteristic of mutual stimulation of transport. The synthetic peptide series did not stimulate vinblastine transport; the various peptides either acted as weak inhibitors of vinblastine uptake or had little effect. Vinblastine also exhibited the expected competitive in-

hibitory effect on peptide transport, decreasing the uptake of NAc-LLY-amide in a concentration-dependent manner.

We cannot exclude the possibility of formation of a complex between colchicine and the synthetic peptides, and the enhanced transport of this complex, as a possible explanation for the results. However, as shown in Figure 8, in addition to colchicine, the chemosensitizers verapamil and cyclosporin A also result in enhanced peptide transport. These three compounds are entirely unrelated structurally, and it seems highly unlikely that simple linear peptides would form a specific complex with each of these molecules. Because each of these compounds is known to interact specifically with P-glycoprotein, a much more likely explanation is the existence of interactions between the sites at which they bind.

None of the members of the hydrophobic peptide series was able to inhibit azidopine photoaffinity labelling of P-glycoprotein or to block the stimulation of ATPase activity caused by verapamil. This is perhaps not unexpected because we have previously shown that linear peptides such as ALLN, ALLM, leupeptin and pepstatin A were also unable to block photolabelling or to abrogate verapamil stimulation [16]. These results were interpreted in terms of separate, non-overlapping sites for binding of linear peptides, and azidopine or verapamil. Thus the binding site for the series of hydrophobic peptides used in this study also does not seem to overlap or interact with the azidopine or verapamil sites. The present results strengthen the idea that not all P-glycoprotein substrates can be identified by their ability to block azidopine photolabelling.

Peptides in the series NAc-L_nY-amide stimulated P-glycoprotein ATPase, but to modest levels compared with some other peptides and ionophores for which increases in catalytic activity of 2.5–3-fold were noted [16,17]. An investigation of the kinetic basis for stimulation of highly purified P-glycoprotein ATPase by various drugs and chemosensitizers, performed in our laboratory, has indicated that they behave as 'mixed' activators, i.e. changes in both K_m and V_{max} were observed [34]. Although the kinetic basis for the increase in ATPase activity induced by peptides remains to be examined, similar changes in kinetic parameters can be expected.

We have previously shown that the actual degree of stimulation of ATPase activity does not correlate well with the 'affinity' of a compound for P-glycoprotein. Rather, the concentration of a compound required for half-maximal stimulation of P-glycoprotein ATPase seems to be a much better correlate of its ability to block drug transport [16]. In the present study the penta- and hexa-peptides showed half-maximal ATPase stimulation concentrations in the range 2–6 μ M, whereas the tri- and tetra-peptides required higher concentrations, approx. 30 μ M. In other words the longer peptides produce higher ATPase stimulation at lower concentrations, yet have the smallest effects on colchicine transport. This behaviour is difficult to interpret because three of these peptides enhance, rather than inhibit, transport. However, there is clearly no simple relationship between ATPase stimulation and stimulation of drug transport. This suggests that communication between the colchicine and linear peptide binding/transport sites does not involve the ATPase domain. Occupation of the peptide site might affect k_{cat} for colchicine transport (and vice versa) without involvement in the energy-coupling step.

The addition of hydrophobic peptides to the rapidly increasing catalogue of known P-glycoprotein substrates has provided an additional insight into the complex interactions between substrates, both negative and positive, that can occur within the P-glycoprotein molecule. The findings of the present study suggest the possibility that such peptides might be one of the physiological substrates for the multidrug transporter, and indicate that

peptide-based chemosensitizers could be useful clinically. Sarkadi and co-workers [17] have already shown that hydrophobic peptides can block P-glycoprotein-mediated drug efflux in intact MDR cells, and animal studies *in vivo* with chemosensitizing peptides are in progress. Characterization of the molecular details of peptide–drug interactions with P-glycoprotein will be necessary to exploit the use of this class of compounds as clinical chemosensitizing agents.

P-Glycoprotein is expressed at the apical surface of the capillary endothelial cells that form the blood/brain barrier, where it seems to play a major role in exclusion of many drugs from the brain. Studies have shown that the sensitivity of homozygous *mdr3*-knockout mice to the neurotoxicity of ivermectin and vinblastine is increased 100-fold compared with wild-type animals [35]. The use of peptides as therapeutic agents has been greatly hindered by their very low penetration across the blood/brain barrier. Chikale et al. [36] have recently obtained evidence for the existence of a verapamil-sensitive apically polarized efflux system for small hydrophobic peptides in the blood/brain barrier of the rat. The model peptides they used [N_{Ac}-F_n-amide, N_{Ac}-F_m(NMeF)_n-amide and N_{Ac}-(NMeF)_n-amide, *m, n* = 1–3] are remarkably similar to those used in the present study, which strongly supports their proposal that such peptide efflux is mediated by P-glycoprotein. Thus peptide transport by P-glycoprotein might play an important role in greatly reducing the delivery of biologically active peptides to the central nervous system *in vivo*. Further studies of the interaction of P-glycoprotein with peptides might lead to strategies to increase peptide transport across the blood/brain barrier.

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