Binding of Steroid Modulators to Recombinant Cytosolic Domain from Mouse P-Glycoprotein in Close Proximity to the ATP Site†

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ABSTRACT: We recently found that recombinant NBD1 cytosolic domain corresponding to segment 395−581 of mouse *mdr*1 P-glycoprotein bound fluorescent 2′(3′)-*N*-methylanthraniloyl-ATP (MANT-ATP) with high affinity [Dayan, G., Baubichon-Cortay, H., Jault, J.-M., Cortay, J.-C., Deléage, G., & Di Pietro, A. (1996) *J. Biol. Chem. 271*, 11652−11658]. The present work shows that a longer 371−705 domain (extended-NBD1), including tryptophan-696 as an intrinsic probe, which bound MANT-ATP with identical affinity, also interacted with steroids known to modulate anticancer drug efflux from P-glycoprotein-positive multidrug-resistant cells. Progesterone, which is not transported, its hydrophobic derivatives medroxyprogesterone acetate and megestrol acetate, and Δ^6-progesterone produced nearly a 50% saturating quenching of the domain intrinsic fluorescence, with dissociation constants ranging from 53 to 18 μM. The even more hydrophobic antiprogestin RU 486 produced a complete quenching of tryptophan-696 fluorescence, in contrast to more hydrophilic derivatives of progesterone containing hydroxyl groups at positions 11, 16, 17, and 21 and known to be transported, which produced very little quenching. A similar differential interaction was observed with full-length purified P-glycoprotein. The steroid-binding region within extended-NBD1 appeared distinct from the nucleotide-binding site as the RU 486-induced quenching was neither prevented nor reversed by high ATP concentrations. In contrast, MANT-ATP binding was efficiently prevented or displaced by RU 486, suggesting that the hydrophobic MANT group of the bound nucleotide analogue overlaps, at least partially, the adjacent steroid-binding region revealed by RU 486.

Multidrug resistance of cancer cells is often associated with overexpression of P-glycoprotein, a membrane transporter that extrudes chemotherapeutic drugs using ATP hydrolysis as the energy source (*1*−*3*). The protein is encoded by the *mdr* gene family comprising two members in man, *MDR*1 and *MDR*2, or three in mouse, *mdr*1 (or *mdr*1b), *mdr*2, and *mdr*3 (or *mdr*1a). Both *mdr*1 and *mdr*3 can convey cellular multidrug resistance in transfection studies as opposed to *mdr*2. The *mdr*2 product appears to be involved in selective translocation of phosphatidylcholine (*4, 5*), whereas *mdr*1 P-glycoprotein may act on a very broad range of short-chain phospholipids (*6*), in addition to many other amphipathic compounds. The relative abundance of *mdr*1 P-glycoprotein in mouse pregnant uterus and adrenal glands (*7*) has suggested a possible involvement in the secretion of steroid hormones: indeed, most steroids including cortisol, dexamethasone, corticosterone, or aldosterone are transported by P-glycoprotein (*8*−*11*). In contrast, progesterone which also interacts with P-glycoprotein (*12, 13*) is not transported (*8, 14*) and behaves as an efficient modulator of cellular multidrug resistance by inhibiting anticancer drug binding and efflux (*11, 12, 14, 15*). Megestrol acetate (*16, 17*) and the antiprogestin RU 486 (*18, 19*) were found to be even more potent than progesterone in reversing the multidrug-resistance phenotype of cancer cells. Steroids also bind to related yeast multidrug transporters of the ABC (ATP-binding cassette)[1] superfamily as they can be transported by PDR5 (*20*) and can inhibit its ATP-dependent drug-pumping activity (*21*); they also seem to interact with SNQ2 (*22*). In addition to a series of drugs, P-glycoprotein is able to bind a number of modulators, most of which are transported (*3*). Several modulator sites have been proposed from either affinity labeling (*23*) or modifications of ATPase activity (*24*) or anticancer drug efflux (*25*), and discrete mutations in membrane domains alter interactions with both anticancer

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[1] Abbreviations, ABC, ATP-binding cassette; ALDP, adrenoleukodystrophy protein; CFTR, cystic fibrosis transmembrane conductance regulator; dialysis buffer, 50 mM potassium phosphate, 100 mM sodium sulfate, 20% glycerol, 0.01% HECAMEG, pH 9.0; HECAMEG, 6-*O*-[(*N*-heptylcarbamoyl)methyl]-α-D-glucopyranoside; IPTG, isopropyl 1-thio-*β*-D-galactopyranoside; MANT-ATP, 2′(3′)-*N*-methylanthraniloyl-ATP; MDR, multidrug resistance; NBD, nucleotide-binding domain; NBD1, *N*-terminal nucleotide-binding domain (from Asn-395 to Thr-581); extended-NBD1, extended *N*-terminal nucleotide-binding domain (from Glu-371 to Glu-705); NBD2, *C*-terminal nucleotide-binding domain; Δ^6-progesterone, 3,20-dioxopregna-4,6-diene; PCR, polymerase chain reaction; PDR5 and SNQ2, yeast multidrug transporters; PMP70, 70-kDa peroxisomal membrane protein; SDS, sodium dodecylsulfate; TAP, transporter associated with antigen processing.

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drugs and modulators (*26, 27*). However, the differential effects produced on different transported drugs or modulators suggest the involvement of a cytosolic interaction in addition to membrane transport and efflux (*26, 28, 29*).

Structural analysis of the P-glycoprotein sequence identifies a polypeptide of 1276 amino acids in mouse (*30*), predicts two homologous halves each containing six putative membrane-spanning α-helices (*30*), as further experimentally confirmed (*31, 32*), and a cytoplasmic nucleotide-binding domain (NBD) with characteristic Walker motifs A and B (*33*); both halves are separated by a central linker region with several putative phosphorylation sites for protein kinase C (*34*). P-Glycoprotein structural organization is typical of the ABC transporter superfamily including mammalian multidrug-resistance-associated protein (*35*) and multidrug transporters from yeasts (*36*) and protozoan parasites (*37*), as well as other eukaryotic transporters acting on peptides [TAP1/TAP2 (*38*) and Ste6 (*39, 40*)], small ions [CFTR (*41*)], or acyl-CoA conjugates [ALDP (*42*) and PMP70 (*43*)]. A recent low-resolution structure for P-glycoprotein indicated the existence of a large hydrophilic membrane pore with an essential gating role played by cytosolic domains (*44*).

An alternative approach to study the properties of cytosolic domains of the transporter and their interactions with ligands is to work with recombinant NBDs, predicted to be soluble, overexpressed in bacteria and purified. The *C*-terminal NBD2 domain was obtained in fusion with glutathione *S*-transferase (*45*) or maltose-binding protein (*46*), and a short *N*-terminal NBD1 domain, corresponding to segment 395−581, was produced as a hexahistidine-tagged protein (*47*). Fluorescence approaches showed that the latter recombinant domain efficiently bound ATP and analogues, but the lack of any sensitive intrinsic probe did not allow the monitoring of possible interactions with other types of substrate or effector.

The aim of the present work was to generate a higher-size *N*-terminal nucleotide-binding domain, namely, extended-NBD1, corresponding to the 371−705 segment including a tryptophan residue, at position 696, which could be used as a powerful intrinsic reporter. The results show that extended-NBD1, which bound nucleotides with the same affinity as shorter NBD1, also contained a cytosolic region able to bind hydrophobic steroid modulators, as evidenced by the high saturating quenching of its intrinsic fluorescence. This newly identified region appeared distinct from, but adjacent to, the ATP-binding site.

EXPERIMENTAL PROCEDURES

Materials. The oligonucleotide primers were purchased from Bioprobe, and the Ni^{2+}-nitrilotriacetic acid agarose gel was from Qiagen. Guanidine hydrochloride was from Pierce. MANT-ATP was obtained as previously described (*47, 48*). IPTG (isopropyl 1-thio-β-D-galactopyranoside), HECAMEG [6-*O*-[(*N*-heptylcarbamoyl)methyl]-α-D-glucopyranoside], and ATP were purchased from Boehringer Mannheim. *N*-Acetyltryptophanamide, dimethyl sulfoxide, and most steroids came from Sigma. Δ^6-Progesterone was synthesized as described (*49*), and RU 486 was a kind gift from Dr. D. Philibert, Roussel-Uclaf (Romainville, France).

Construction of Expression Vectors. The cDNA encoding mouse *mdr*1 was obtained as described (*50*). PCR synthesis of the cDNA encoding extended-NBD1 was performed as previously detailed for NBD2 (*45*) and NBD1 (*47*). Here, the two primers specific to *N*-terminal Glu-371 and *C*-terminal Glu-705 of extended-NBD1 were respectively: 5′ TATGAATTCACGAGCCAAGCATTGACAGCT 3′ and 5′ TATCTGCAGTTCACTTAGATTTAGATTTAGGAT 3′.

The primers allowed the introduction of *Eco*RI and *Pst*I restriction sites, and the 1105-bp amplified cDNA was digested by endonucleases and ligated into the corresponding sites of linearized pT7-7 plasmid. The latter was kindly provided by Dr. J.-C. Cortay: it was modified to include the hexahistidine-coding sequence between the *Pst*I and *Hin*dIII sites of the multiple cloning site, as described for the pT7-5 plasmid (*51*). *Escherichia coli* BL21(DE3) cells [F^- *dcm ompT hsdS*(r_{B^-} m_{B^-}) *gal* λ(DE3)] were transformed with the ligation product and grown on agar plates supplemented with ampicillin (50 μg/mL).

Overexpression, Domain Renaturation, and Purification. E. coli cells harboring the appropriate recombinant plasmid were grown at 37 °C in LB medium [1% (w/v) bactotryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl] at pH 7.5 containing 50 μg of ampicillin/mL, until the absorbance at 600 nm reached 0.7 unit. Expression of the recombinant domain was induced with 2 mM IPTG for 2 h at 37 °C. Cell harvesting and lysis by French press treatment were as described earlier for shorter NBD1 (*47*).

The pellet from centrifugation at 30000*g* for 30 min was suspended in 50 mM Tris-HCl, pH 7.5, containing 1 mM ethylenediaminetetraacetic acid, 6 mM $MgCl_2$, 0.4 M NaCl, and 1% Triton X100. The recombinant domain was then solubilized by 1.5 M guanidine with vigorous stirring and incubation for 30 min at 30 °C. The supernatant obtained from centrifugation at 30000*g* for 20 min was quickly diluted with 20 volumes of 50 mM potassium phosphate, pH 9.0, containing 100 mM sodium sulfate and 20% glycerol. After centrifugation at 30000*g* for 20 min, the renaturated domain was purified through a Ni^{2+}-nitrilotriacetic acid column as previously described for NBD1 (*46*). The fractions eluted with 200 mM imidazole were pooled and dialyzed against 50 mM potassium phosphate, 100 mM sodium sulfate, 20% glycerol, 0.01% HECAMEG (dialysis buffer) at pH 9.0. The dialysate (0.23−0.35 mg of protein/mL) was aliquoted and kept frozen in liquid nitrogen. Protein fractions were analyzed on 12% polyacrylamide SDS gels as described by Laemmli (*52*). Protein concentration was routinely determined by the method of Bradford (*53*) with the Coomassie blue Plus Protein Assay Reagent kit from Pierce.

Highly purified, full-length P-glycoprotein was obtained from plasma membranes of mouse P388/ADR25 cells as recently described (*54*).

Fluorescence. Experiments were performed at 25.0 ± 0.1 °C using a SLM-Aminco 8000C spectrofluorometer with spectral bandwidths of 2 and 4 nm respectively for excitation and emission. The measurements were corrected for wavelength dependence on the excitation light intensity by using rhodamine B in the reference channel. All spectra were corrected for buffer fluorescence and for dilution.

Fluorescence measurements were performed by diluting extended-NBD1 (1−3 μM final concentration) in 1 mL of dialysis buffer, at pH 9.0, in the presence of increasing concentrations of nucleotides or dimethyl sulfoxide solutions of steroids. Tryptophan intrinsic fluorescence was studied on 1 μM extended-NBD1 by scanning emission in the range 310−380 nm upon excitation at 295 nm. The binding of

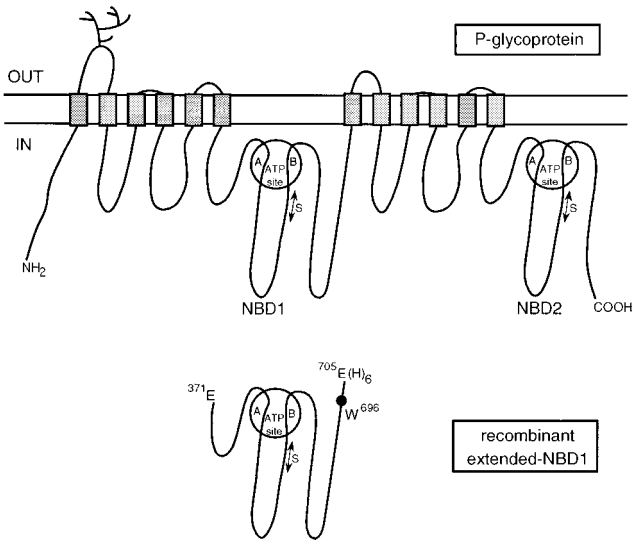

FIGURE 1: Schematic structures of mouse full-length P-glycoprotein and recombinant extended-NBD1 cytoplasmic domain. Top: Topological model of P-glycoprotein showing the putative transmembrane helices (boxes) and the two cytoplasmic nucleotide-binding domains, NBD1 and NBD2, each containing the characteristic Walker motifs A and B of the ATP site and the ABC transporter signature (S). Bottom: Recombinant, hexahistidine-tagged extended-NBD1 domain lying from Glu-371 to Glu-705 and containing a single tryptophan residue at position 696.

nucleotides or steroids was monitored by the quenching of emission fluorescence produced by addition of increasing concentrations. Corrections for inner-filter effect and dimethyl sulfoxide (up to 2% final concentration) were determined under the same conditions using *N*-acetyltryptophanamide. Curve fitting of nucleotide or steroid binding was performed with Grafit (Erithacus software) as described previously (*45*, *47*, *55*). Fluorescence resonance energy transfer between tryptophan-696 of extended-NBD1 and bound MANT-ATP was monitored by the appearance of a fluorescence emission peak between 420 and 460 nm, characteristic of bound nucleotide analogue, upon tryptophan excitation at 295 nm.

Full-length purified P-glycoprotein was diluted to 23 nM final concentration in 12% sucrose, 10 mM Tris-HCl, pH 7.5, 1 mM ethylenediaminetetraacetic acid, 0.02% *n*-dodecyl β-D-maltoside.

When the extrinsic fluorescence of MANT-ATP was studied, excitation was performed at 350 nm. The nucleotide analogue binding was determined from the increase in fluorescence between 420 and 480 nm, in the presence as compared to the absence of extended-NBD1. Curve fitting of the concentration-dependent analogue binding was performed with Grafit as described previously (*45*, *47*, *55*). For RU 486-dependent displacement of bound MANT-ATP, controls were conducted using the same concentrations of steroid and ATP analogue but in the absence of protein.

RESULTS

Overexpression and Purification of the Cytosolic Extended-NBD1 Domain from Mouse P-Glycoprotein. From current models of P-glycoprotein based on cDNA sequence and predictions from hydrophobicity profiles (*1*, *2*), the two nucleotide-binding domains (NBD1 and NBD2) are assumed to be cytosolic (Figure 1, upper scheme); they both contain three consensus sequences: the Walker motifs A and B involved in ATP binding and the ABC transporter signature S (*56*). The aim of the present work was to obtain a cytosolic extended-NBD1 domain (lower scheme), from glutamate-371 to glutamate-705, which is predicted to be located just before the first transmembrane span of the second membrane domain. For this purpose, the corresponding cDNA was produced by PCR and inserted into the modified pT7-7 vector for overexpression in *E. coli* cells and affinity purification by means of a *C*-terminal hexahistidine tag. Recombinant extended-NBD1 contains a single tryptophan residue, located at position 696.

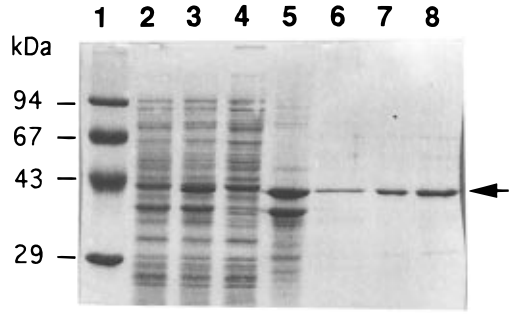

FIGURE 2: Overexpression, renaturation from inclusion bodies, and purification of the extended-NBD1 domain. Fractions obtained from a 1-L culture of *E. coli* cells overexpressing the cDNA encoding extended-NBD1 were analyzed by SDS−polyacrylamide gel electrophoresis: lane 1, molecular weight markers corresponding to phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), and carbonic anhydrase (29 kDa); lane 2, total bacteria proteins before IPTG induction (loaded sample equivalent to 130-μL culture); lane 3, total bacteria proteins after IPTG induction (sample equivalent to 90-μL culture); lane 4, soluble proteins from the supernatant; lane 5, inclusion bodies recovered in the pellet; lane 6, inclusion bodies solubilized with 1.5 M guanidine and renatured, after centrifugation, by quick dilution with 20 volumes of buffer at pH 9.0; lanes 7 (1 μg of protein) and 8 (2.5 μg of protein), purified domain obtained after nickel chelate affinity chromatography and dialysis. The arrow indicates the position of recombinant extended-NBD1 (38.9 kDa).

Figure 2 shows that two main bacterial proteins with apparent molecular weights of 42 and 37 kDa were present before induction (lane 2). IPTG induction highly overexpressed the recombinant extended-NBD1 domain which migrated just below the 42-kDa component (lane 3). However, the recombinant domain was absent from the soluble fraction which mainly contained the 42-kDa protein (lane 4); the domain was exclusively recovered as inclusion bodies in the pellet of centrifugation together with the 37-kDa component (lane 5). A rather low guanidine concentration, 1.5 M, was sufficient to solubilize extended-NBD1 from inclusion bodies, whereas the major 37-kDa contaminant remained insoluble (lane 6). The domain was renatured by quick dilution with 20 volumes of phosphate buffer at pH 9.0, containing glycerol and high salt concentration, and loaded onto the nickel-agarose column for final purification. The retained fraction was then eluted with 200 mM imidazole and dialyzed in 50 mM potassium phosphate, 100 mM sodium sulfate, 20% glycerol, 0.01% HECAMEG, at pH 9.0 (lanes 7 and 8). Up to 5 mg of protein of purified domain, at a 6−9 μM concentration, could be obtained from a 1-L culture.

The apparent molecular mass of the domain appeared slightly higher than the theoretical value, 38.9 kDa, as also previously observed for other hexahistidine-tagged proteins (*47*). The intrinsic fluorescence spectrum of purified extended-

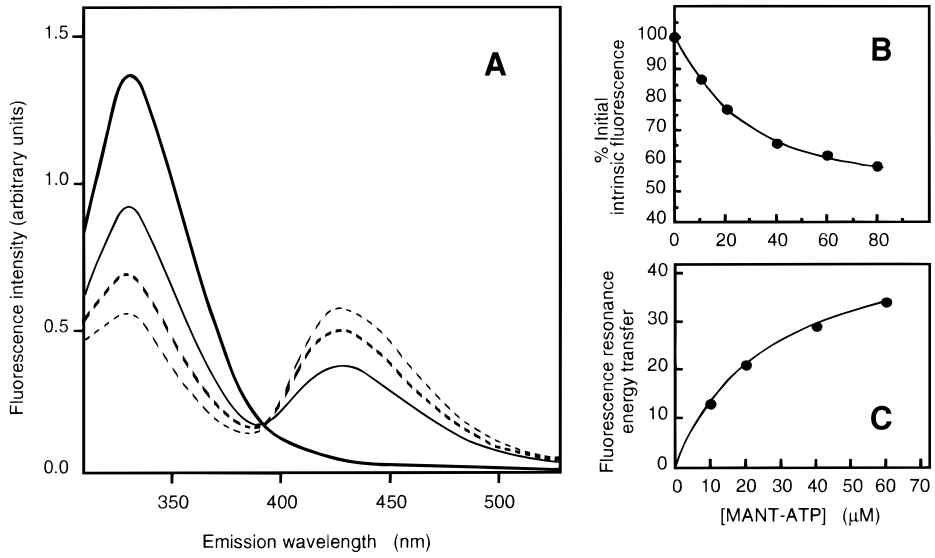

FIGURE 3: MANT-ATP binding to extended-NBD1 as monitored by quenching of intrinsic fluorescence and fluorescence resonance energy transfer. A: Spectral modifications upon interaction of extended-NBD1 with MANT-ATP. The fluorescence spectrum of 2 μM extended-NBD1 was recorded after excitation at 295 nm in 1 mL of dialysis buffer, at pH 9.0, in the absence of MANT-ATP (thick solid trace) or in the presence of 20 (thin solid trace), 40 (thick dashed trace), or 60 (thin dashed trace) μM concentrations; the traces were obtained by buffer subtraction before correction for inner-filter effect. The concentration-dependent binding of MANT-ATP was analyzed by both the quenching of extended-NBD1 intrinsic fluorescence, determined by integration from 310 to 380 nm and corrected for inner-filter effect (B), and the fluorescence increase of bound MANT-ATP between 400 and 530 nm (C).

NBD1 indicated that the single tryptophan residue, at position 696, exhibited a hydrophobic environment as a low wavelength for maximal emission, 325 nm, was observed upon excitation at 295 nm (Figure 3A, thick solid trace). A considerable red shift was produced upon denaturation of the protein by overnight incubation with 6 M guanidine, leading to a maximal emission wavelength of 348−350 nm comparable to that observed with *N*-acetyltryptophanamide (not shown here).

Interactions with Nucleotides and Steroids. Addition of the fluorescent nucleotide analogue MANT-ATP to purified extended-NBD1 produced both quenching of the domain intrinsic fluorescence and concomitant increase in a new fluorescence peak with a maximum at 432 nm: this was characteristic for fluorescence resonance energy transfer between tryptophan-696 and the MANT group of bound MANT-ATP (Figure 3A, dashed and thin solid traces as compared to thick solid trace). The spectral modifications showed a saturating dependence on the analogue concentration, and curve fitting using Grafit allowed determination of very similar dissociation constant values for MANT-ATP: 27.2 ± 2.5 μM from quenching of intrinsic fluorescence (Figure 3B) and 24.3 ± 1.6 μM from fluorescence resonance energy transfer (Figure 3C). A maximal quenching value of 57 ± 2% was determined from Figure 3B.

Incubation of purified extended-NBD1 with steroids also led to significant quenching of the domain intrinsic fluorescence, but the extent of quenching was strongly dependent on the hydrophobicity of the compound (Figure 4). Indeed, progesterone, a hydrophobic nontransported steroid, and Δ^6-progesterone produced a marked quenching with a maximal value of 48 ± 3% and a dissociation constant of 53 ± 5 or 27 ± 3 μM, respectively. Addition of an aromatic ring at position 11, in the antiprogestin RU 486, increased both the maximal extent of quenching to 98 ± 2% and the affinity (K_d = 20 ± 2 μM). In contrast, a very low effect was observed with more hydrophilic derivatives, such as

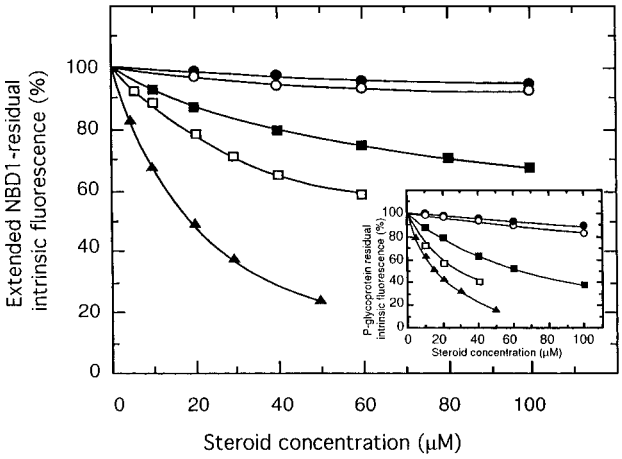

FIGURE 4: Interaction with steroids as monitored by quenching of protein intrinsic fluorescence. Extended-NBD1 was assayed under conditions of Figure 3, in the presence of increasing concentrations of the following steroids as dimethyl sulfoxide solutions: triamcinolone (●), cortisol (○), progesterone (■), Δ^6-progesterone (□), or RU 486 (▲). The peak of intrinsic fluorescence emission was integrated between 310 and 360 nm and corrected for inner-filter effects, dimethyl sulfoxide, and buffer fluorescence. Maximal quenching values and dissociation constants were determined using Grafit. Inset: Same steroid-binding experiments but using full-length P-glycoprotein instead of extended-NBD1 (see Experimental Procedures).

cortisol and triamcinolone, which are known to be transported substrates for P-glycoprotein. The inset of Figure 4 indicates that full-length purified P-glycoprotein behaved very similarly to extended-NBD1 toward steroid interaction, with comparable affinities and the same preference, RU486 > Δ^6-progesterone > progesterone > cortisol > triamcinolone, although the maximal quenching values were somewhat higher.

Since a K_d value could not be accurately determined for hydrophilic steroids producing low quenching at routinely used concentrations, screening of steroid substituent effects

Table 1: Role of Steroid Substituents on the Quenching of Extended-NBD1 Intrinsic Fluorescence[a]

steroid	substituents						% quenching at 50 μM[b]
	6	11	16	17	20/21	double bond	
progesterone					$COCH_3$	4	23
11α-hydroxyprogesterone		OH			$COCH_3$	4	7
17α-hydroxyprogesterone				OH	$COCH_3$	4	9
11-deoxycorticosterone					$COCH_2OH$	4	17
corticosterone		OH			$COCH_2OH$	4	6
cortisol		OH		OH	$COCH_2OH$	4	5
dexamethasone		OH	CH_3	OH	$COCH_2OH$	1, 4	8
triamcinolone		OH	OH	OH	$COCH_2OH$	1, 4	3
triamcinolone acetonide		OH	O-C$(CH_3)_2$-O		$COCH_2OH$	1,4	10
testosterone			OH			4	8
dihydrotestosterone			OH				5
5β-pregnanedione					$COCH_3$		18
Δ^6-progesterone					$COCH_3$	4, 6	37
medroxyprogesterone acetate	CH_3			$OCOCH_3$	$COCH_3$	4	27
megestrol acetate	CH_3			$OCOCH_3$	$COCH_3$	4, 6	45
RU 486	Ph-N$(CH_3)_2$			OH	C_2-CH_3	4, 9	75

[a] The extended-NBD1 domain was incubated, under conditions of Figure 4, with progesterone or a series of derivatives up to 100 μM concentrations. [b] The quenching of intrinsic fluorescence was plotted as a function of concentration, and the quenching produced at 50 μM was graphically determined.

was performed by measuring the extent of quenching of extended-NBD1 intrinsic fluorescence produced by a fixed, 50 μM, steroid concentration (Table 1). The results extended the observations of Figure 4 and led to the following structure−activity relationships: (i) Increased hydrophilicity due to increased number of hydroxyl groups dramatically lowered the quenching efficiency, with a preference for positions 11, 16, and 17 as compared to 21 and with overall additive effects, when comparing the first group of hydroxylated derivatives; (ii) a double bond at position 4 appeared to be involved in quenching since its absence in dihydrotestosterone and 5β-pregnanedione was associated with lower effects compared respectively to testosterone and progesterone; (iii) reciprocally, an additional double bond at position 6 markedly increased quenching when comparing Δ^6-progesterone and megestrol acetate respectively to progesterone and medroxyprogesterone acetate.

Figure 5A shows that the RU 486-induced quenching of extended-NBD1 intrinsic fluorescence (open symbols) was not significantly modified by preincubation with 10 mM ATP (closed symbols). Bound RU 486 was not displaced either by nucleotide addition as the 50% quenching produced by incubation with 20 μM RU 486 was not removed by increasing ATP concentrations up to 20 mM (not shown here).

In contrast, the binding of MANT-ATP, as monitored by enhancement of extrinsic fluorescence and giving a K_d value of 27 ± 3 μM (Figure 5B, open symbols), was highly inhibited by preincubation with a 50 μM RU 486 concentration (closed symbols). In addition, initially bound MANT-ATP (nearly 0.5 mol/mol upon incubation with a 25 μM concentration) was efficiently and gradually displaced, as followed by a decrease in extrinsic fluorescence intensity, upon addition of increasing RU 486 concentrations (inset of Figure 5B). Therefore, MANT-ATP and RU 486 exhibited mutually exclusive binding.

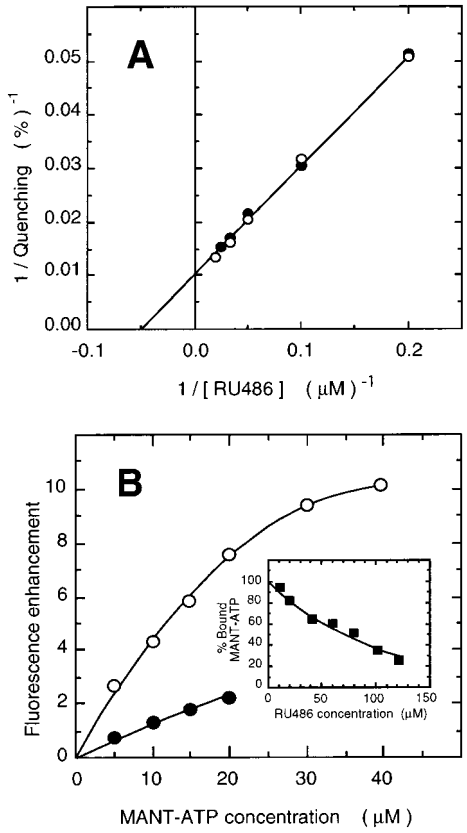

FIGURE 5: Antagonistic binding effects between RU 486 and nucleotides. A: Extended-NBD1 was preincubated (●) or not (○) with 10 mM ATP and then assayed for quenching of intrinsic fluorescence, under conditions of Figure 4, by addition of increasing RU 486 concentrations. B: Concentration-dependent binding of MANT-ATP to extended-NBD1 was monitored, after preincubation in the absence (○) or presence (●) of 50 μM RU 486, by the increase in extrinsic fluorescence between 420 and 460 nm upon excitation at 350 nm. Inset: Displacement of MANT-ATP, bound from incubation with a 25 μM concentration, by addition of increasing RU 486 concentrations.

DISCUSSION

Overexpression and purification of the extended-NBD1 domain from P-glycoprotein have allowed here to demonstrate the existence of a cytosolic region able to bind hydrophobic steroid modulators. This region appears distinct from, but adjacent to, the ATP site.

Recombinant Extended-NBD1 Domain: Solubility and Nucleotide Binding. This paper describes overexpression and purification of a cytosolic nucleotide-binding domain from P-glycoprotein including the sequence preceding the second membrane domain. The entire cytosolic domain appeared

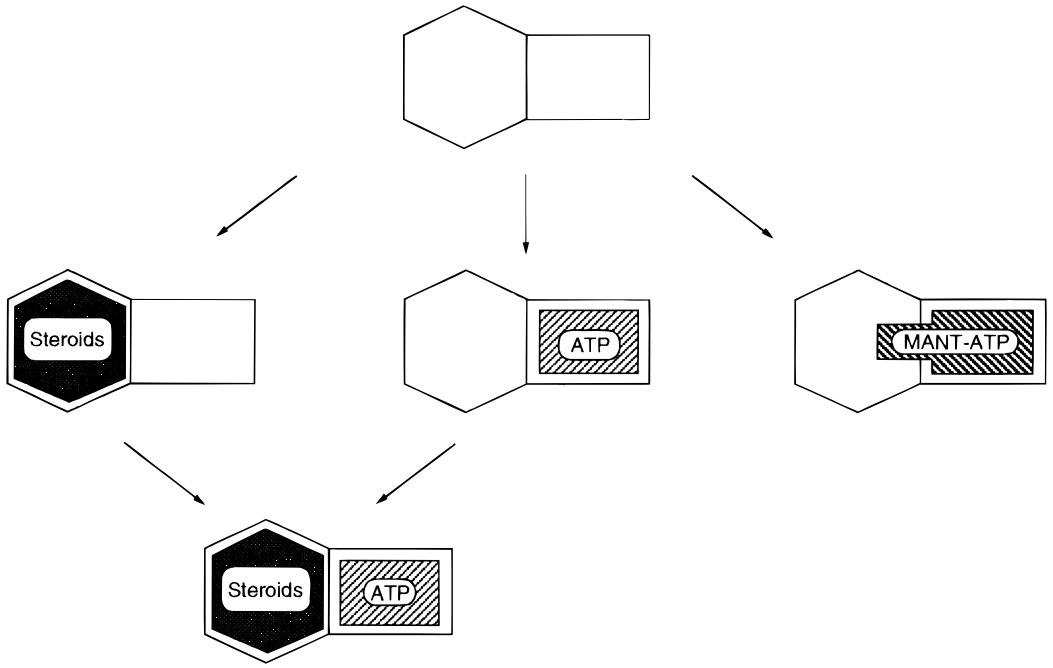

FIGURE 6: Tentative model of adjacent nucleotide- and steroid-binding regions within a cytosolic domain of P-glycoprotein. The results obtained in the present work suggest the existence of a cytosolic steroid-binding region (hexagon) adjacent to the ATP site (rectangle) in purified extended-NBD1 domain. Steroids and ATP are able to bind, either separately or together. In contrast, the mutually exclusive binding of the nucleotide analogue MANT-ATP and RU 486 indicates that the hydrophobic MANT group would overlap the neighboring steroid-binding region.

quite hydrophobic and was recovered as insoluble inclusion bodies in contrast to *C*-terminal NBD2 (*45*, *46*) and shorter NBD1 (*47*). In the latter case, it was clearly shown that increasing the *C*-side extension, from threonine-581 to glutamate-613 or serine-643, gradually lowered the protein solubility during overexpression. The poor solubility of the present domain which is extended up to glutamate-705 strengthens the previous observations, although a number of charged residues are present in the linker region.

Despite its lower solubility, renatured extended-NBD1 behaves very similarly to NBD1 toward nucleotide binding. Indeed, a 24−27 μM K_d value for MANT-ATP was obtained from either quenching of (tryptophan) intrinsic fluorescence, fluorescence resonance energy transfer between tryptophan-696 and the MANT group of bound nucleotide analogue, or enhancement of MANT-ATP extrinsic fluorescence; this K_d value was identical with that obtained with shorter NBD1 by quenching of either the less-sensitive tyrosine fluorescence or the MANT-ATP extrinsic fluorescence (*47*). Finally, the same renaturation procedure, involving solubilization of inclusion bodies with 1.5 M guanidine and quick dilution with 20 volumes of phosphate buffer, at alkaline pH, containing glycerol and high salt concentration, was successfully applied to shorter NBD1: the renatured form exhibited identical properties toward nucleotides and nucleotide analogues as compared with the protein directly recovered from the soluble fraction (*47*). A related procedure was also efficiently used for renaturing an active recombinant domain from the α-subunit of mitochondrial ATPase (*57*).

Tryptophan-696 exhibited a quite hydrophobic environment with a low wavelength for maximal emission, 325 nm, which was markedly red-shifted (23−25 nm) upon denaturation with 6 M guanidine. This feature further confirms an accurate folding of renatured extended-NBD1, at least around tryptophan-696. Tryptophan-696 appears quite distant from the ATP site: an approximate distance of 17−23 Å may be estimated with respect to the MANT group of bound nucleotide analogue on the basis of a value of 57% for maximal resonance energy-transfer efficiency and assuming a Förster distance value of 20−25 Å, as generally observed for tryptophan and MANT as a donor/acceptor pair (*58*).

Cytosolic Binding Region Interacting with Steroid Modulators. P-Glycoprotein is shown here to be highly quenched upon binding progesterone, a modulator of MDR cellular phenotype which is not transported (*8*, *14*); the quenching is even higher with the antiprogestin RU 486, a more efficient modulator (*18*, *19*), whereas a very low quenching is produced by transported steroids like triamcinolone and cortisol (*8*−*11*). Similar progesterone concentrations were reported to stimulate P-glycoprotein ATPase activity when either purified (*59*, *60*) or inserted in plasma membranes (*24*, *61*), to revert cellular MDR phenotype by inhibiting anticancer drug efflux (*11*), and to lower azidopine photoaffinity labeling (*12*).

The fact that a similar affinity binding is observed with the purified cytosolic domain extended-NBD1 strongly indicates that the recombinant domain does not expose an artificial hydrophobic area which is normally buried in the full-length transporter and that steroid binding to P-glycoprotein at least partly concerns its cytosolic domains. The higher maximal quenching observed for the whole transporter might indicate a higher binding (at least 2 mol of steroid/mol of P-glycoprotein) although possible involvement of tryptophan residues from the membrane domains cannot be excluded. We indeed observed an additional steroid binding to recombinant cytosolic domain NBD2 (A. Di Pietro et al., unpublished experiments).

The decreasing affinity binding sequence determined here for steroids toward extended-NBD1 (RU 486, megestrol acetate > Δ^6-progesterone > medroxyprogesterone acetate > progesterone > 5β-pregnanedione, 11-deoxy corticosterone > triamcinolone acetonide, 17α-hydroxyprogesterone,

dexamethasone, testosterone > 11α-hydroxyprogesterone, corticosterone, dihydro-testosterone, cortisol > triamcinolone) correlates directly with their potency as inhibitors of cellular drug efflux and inversely to their ability to be transported by P-glycoprotein. Indeed, RU 486 (*18*, *19*) and megestrol acetate (*16*, *17*) were found to be more than 2-fold better than progesterone in reversing the cellular multidrug-resistance phenotype by inhibiting related anticancer drug efflux. In contrast, triamcinolone, cortisol, corticosterone, testosterone, and dexamethasone, which were shown to be efficiently transported (*8*, *9*, *11*, *14*), behaved as less-efficient modulators of anticancer drug efflux (*14*, *62*). This appeared to be directly related to increased hydrophilicity, especially due to the presence of hydroxyl groups at positions 11, 16, 17, and, to a lower extent, 21 (*10*, *14*). Other modulators known to be efficiently transported like verapamil and cyclosporin A (*3*) also produced a very limited quenching of extended-NBD1 fluorescence (G. Dayan and A. Di Pietro, unpublished data).

We do not know if a membrane site indeed exists for progesterone, as proposed from kinetic experiments (*24*), but the present results clearly demonstrate the existence within extended-NBD1 of a cytosolic region interacting with the nontransported modulator and its hydrophobic derivatives that are able to revert the multidrug-resistance phenotype of cancer cells.

Location of the Cytosolic Steroid-Binding Region. The modulator-binding region appears distinct from the nucleotide-binding site as ATP does not produce any antagonism against RU 486 binding by either prevention or displacement. In contrast, MANT-ATP, whose binding is competitive toward ATP, as shown for shorter NBD1 (*47*), is quite efficient in antagonizing RU 486 binding. This suggests that the hydrophobic MANT group of bound MANT-ATP overlaps the modulator-binding region, which implies that both binding regions are adjacent, as illustrated in the tentative model proposed in Figure 6. Although a distant conformational change could not be completely excluded, it seemed unlikely since it would have been expected to also occur with ATP.

The nearly complete quenching induced by RU 486 suggests that tryptophan-696 might be in close proximity to bound modulator, maybe at the level of the additional aromatic ring branched on position 11. This does not exclude, however, the possible contribution of other domain regions, such as sequences located between Walker motifs A and B.

In conclusion, the present work shows that both ATP and steroid-binding regions are present on the cytosolic extended-NBD1 domain of P-glycoprotein, which constitutes a quite promising material for identifying amino acids involved in both types of binding. Extensive study of this cytosolic steroid-binding region, by combining chemical modifications and site-directed mutagenesis, may facilitate our understanding of the molecular mechanism of ATP-dependent drug efflux and allow design of new, specific inhibitors.

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