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ARTICLE

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Definition of the domain boundaries is critical to the expression of the nucleotide-binding domains of P-glycoprotein

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Abstract Heterologous expression of domains of eukaryotic proteins is frequently associated with formation of inclusion bodies, consisting of aggregated mis-folded protein. This phenomenon has proved a significant barrier to the characterization of domains of eukaryotic ATP binding cassette (ABC) transporters. We hypothesized that the solubility of heterologously expressed nucleotide binding domains (NBDs) of ABC transporters is dependent on the definition of the domain boundaries. In this paper we have defined a core NBD, and tested the effect of extensions to and deletions of this core domain on protein expression. Of 10 NBDs constructed, only one was expressed as a soluble protein in *Escherichia coli*, with expression of the remaining NBDs being associated with inclusion body formation. The soluble NBD protein we have obtained corresponds to residues 386–632 of P-glycoprotein and represents an optimally defined domain. The NBD has been isolated and purified to 95% homogeneity by a two-step purification protocol, involving affinity chromatography and gel filtration. Although showing no detectable ATP hydrolysis, the protein retains specific ATP binding and has a secondary structure compatible with X-ray crystallographic data on bacterial NBDs. We have interpreted our results in terms of homology models, which suggest that the N-terminal NBD of P-glycoprotein can be produced as a stable, correctly folded, isolate domain with judicious design of the expression construct.

Keywords ABC transporter · Modelling · Multidrug resistance · Nucleotide binding domain · P-glycoprotein

Abbreviations ABC: ATP binding cassette · ANOVA: analysis of variance · BRP: bacteriocin release protein · BSA: bovine serum albumin · CD: circular dichroism · CFTR: cystic fibrosis transmembrane conductance regulator · GFP: green fluorescent protein · IPTG: isopropyl-1-thio-β-galactopyranoside · LB: Luria Bertani · LS: low salt LB media · NBD: nucleotide binding domain · Ni-NTA: nickel-nitrilotriacetic acid · PCR: polymerase chain reaction · P-gp: P-glycoprotein · s.e.m.: standard error of the mean · TAP: transporter associated with antigen processing · TMD: transmembrane domain

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Introduction

Members of the ATP binding cassette (ABC) family of membrane transport proteins are present in all organisms and underlie diverse physiological processes, including bacterial nutrient uptake and cancer cell multidrug efflux (Decottignies and Goffeau 1997; Higgins 1992). In humans, defects in ABC transporters are implicated in diseases such as cystic fibrosis (Riordan et al. 1989) and adrenoleukodystrophy (Cartier et al. 1993). The architecture of the majority of ABC transporters consists of two transmembrane domains (TMD) which delineate a pathway for the transported substrate (allocrite), and two nucleotide binding domains (NBD), which couple the energy associated with ATP binding and hydrolysis to allocrite transport (Holland and Blight 1999; Kerr 2002). Prokaryotic NBDs, which are frequently encoded as separate polypeptides, have been characterized following their over-expression. For example, the NBD of the *Salmonella* histidine uptake

transport system (HisP) and the NBD of both mesophilic bacterial and thermophilic archaeal maltose transporters (MalK) have been expressed and characterized as ATPase domains (Greller et al. 1999; Morbach et al. 1993; Nikaido et al. 1997). Detailed biochemical analysis suggests that some of the fundamental catalytic properties of an ABC transporter (such as nucleotide binding) are reflected in the isolated NBD (Davidson et al. 1996; Morbach et al. 1993). Furthermore, several prokaryotic NBDs have had structures determined to high resolution (Diederichs et al. 2000; Hung et al. 1998; Karpowich et al. 2001; Yuan et al. 2001), enabling a structural basis for the conservation of key amino acids to be elucidated (Kerr 2002).

The advances in our understanding of ABC transporters resulting from studies of isolated prokaryotic NBDs have encouraged attempts to investigate biomedically important eukaryotic ABC transporters, such as the multidrug transporter P-glycoprotein (P-gp), by characterization of individual domains. The characterization of isolated NBDs is a prerequisite for employing these domains in studies aimed at elucidating interactions between NBD and NBD, and between NBD and TMD, the exact nature of which is still the subject of debate (Linton et al. 2003). However, despite the predicted cytoplasmic localization of the NBD, similar attempts to express the NBDs of eukaryotic ABC transporters as isolated proteins have met with considerable difficulty, with the notable exception of the NBD of the transporter associated with antigen processing, TAP1 (Gaudet and Wiley 2001). The majority of eukaryotic NBD expression attempts have been characterized by the formation of inclusion bodies. In order to circumvent such difficulties, attempts have been made to refold protein subsequent to the isolation of inclusion bodies (Booth et al. 2000), although this approach is hampered by the requirement to demonstrate that the refolded protein population is homogeneous (Hough et al. 2002). Other groups have facilitated the production of soluble protein by the expression of eukaryotic NBDs fused to carrier proteins such as maltose binding protein (Ko and Pedersen 1995; Roerig et al. 2001; Sharma and Rose 1995; Wilkes et al. 2002) or glutathione S-transferase (Baubichon-Cortay et al. 1994). However, fusion proteins may not be suitable for structural studies unless the protein can be effectively cleaved and purified from its fusion partner (Berridge et al. 2003).

In this paper we have adopted an alternative approach in attempting to express the NBDs of P-gp as soluble proteins (i.e. which remain in the supernatant subsequent to centrifugation at 60,000×*g*). We hypothesize that the domain definition of the NBD is critical to achieving production of soluble protein. By employing sequence alignments and structural information to design alternative definitions of the N- and C-terminal NBD of P-gp, and by optimization of expression conditions, we have produced the N-terminal NBD as a stable protein domain. This domain has a secondary structure characteristic of NBDs that have had been elucidated at high resolution, and retains the ability to bind nucleotide. The characterization of homology models of the NBDs of P-gp enables us to provide hypotheses for the successful expression of the N-terminal NBD.

Materials and methods

Materials

All chemicals were of reagent grade or better and were obtained from Sigma-Aldrich (Poole, UK) or Calbiochem (Nottingham, UK) unless stated. Oligonucleotides were obtained from Sigma-Genosys. Plasmid pET-20 was obtained from Novagen (Nottingham, UK) and pBAD-GFP*uv* was a gift of Dr. M. Blight (Institut de Génétique et Microbiologie, Orsay, France). All restriction enzymes were from New England Biolabs (Hitchin, UK).

Molecular biology

N- and C-terminal NBD constructs (Fig. 1) were amplified by the polymerase chain reaction (PCR), employing oligonucleotide primers as reported in Table 1. Template DNA encoded P-gp from which all cysteines had been removed and replaced by serine residues (Taylor et al. 2001). PCR products were cloned into the pET-20 vector at *Nde*I/*Xho*I sites (N-terminal NBDs) or *Nde*I/*Not*I (C-terminal NBDs). Insertion of PCR products at the *Nde*I site resulted in the addition of an initiation codon (Met) to the N-terminus of the proteins. At the 3′ end, cloning resulted in the addition of Leu-Glu-$(His)_6$ to the C-terminus of NBD-N1 to NBD-N5, and of Ala-Ala-Ala-$(His)_6$ to the C-terminus of NBD-C1 to NBD-C5. Since expression of NBDs from the *lac* operon resulted in inclusion body formation (see Results), the entire coding sequence for the 10 NBDs (including the hexa-histidine tag) was additionally sub-cloned from pET-20 into a vector derived from pBAD-GFP*uv*, which places control of expression of proteins under the *araBAD* operon, such that the GFP cDNA was entirely replaced by the NBD cDNA. Sequences of expression plasmids were verified by DNA sequencing (Department of Biochemistry, University of Oxford). All such expression plasmids are named pBAD-NBD-N1 to pBAD-NBD-C5.

Protein expression

pBAD-derived expression plasmids were transformed into *Escherichia coli* either by heat shock of chemically competent BL21.λDE3, BL21.λDE3.pLysS (Studier et al. 1990) or by electroporation of GJ1158 cells (Bhandari and Gowrishankar 1997). BL21.λDE3 transformants were selected on Luria Bertani (LB) agar plates containing 100 μg/mL ampicillin, and the pLysS derivative on plates further supplemented with 34 μg/mL chloramphenicol. GJ1158 transformants were selected on low salt media (LS, essentially LB broth without added NaCl) supplemented with 100 μg/mL ampicillin. Single colonies of transformed strains were grown overnight in 5 mL of appropriate medium supplemented with antibiotics at 37 °C, before being diluted 1:100 into 400 mL selective media. Growth was continued at 37 °C until an OD_{600} of 0.5 was attained. Cultures were cooled to 20 °C and protein expression was induced by the addition of L-arabinose to a final concentration of 0.02% w/v. Growth was continued at 18–22 °C for a further 6 h. Cells were harvested by centrifugation at 4 °C for 15 min at 4000×*g* (JA-10 rotor), and pellets retained at −20 °C until required.

Protein purification

Bacterial cell pellets were resuspended in lysis buffer (10 mM Tris, 150 mM NaCl, 20% glycerol v/v, pH 7.4) containing

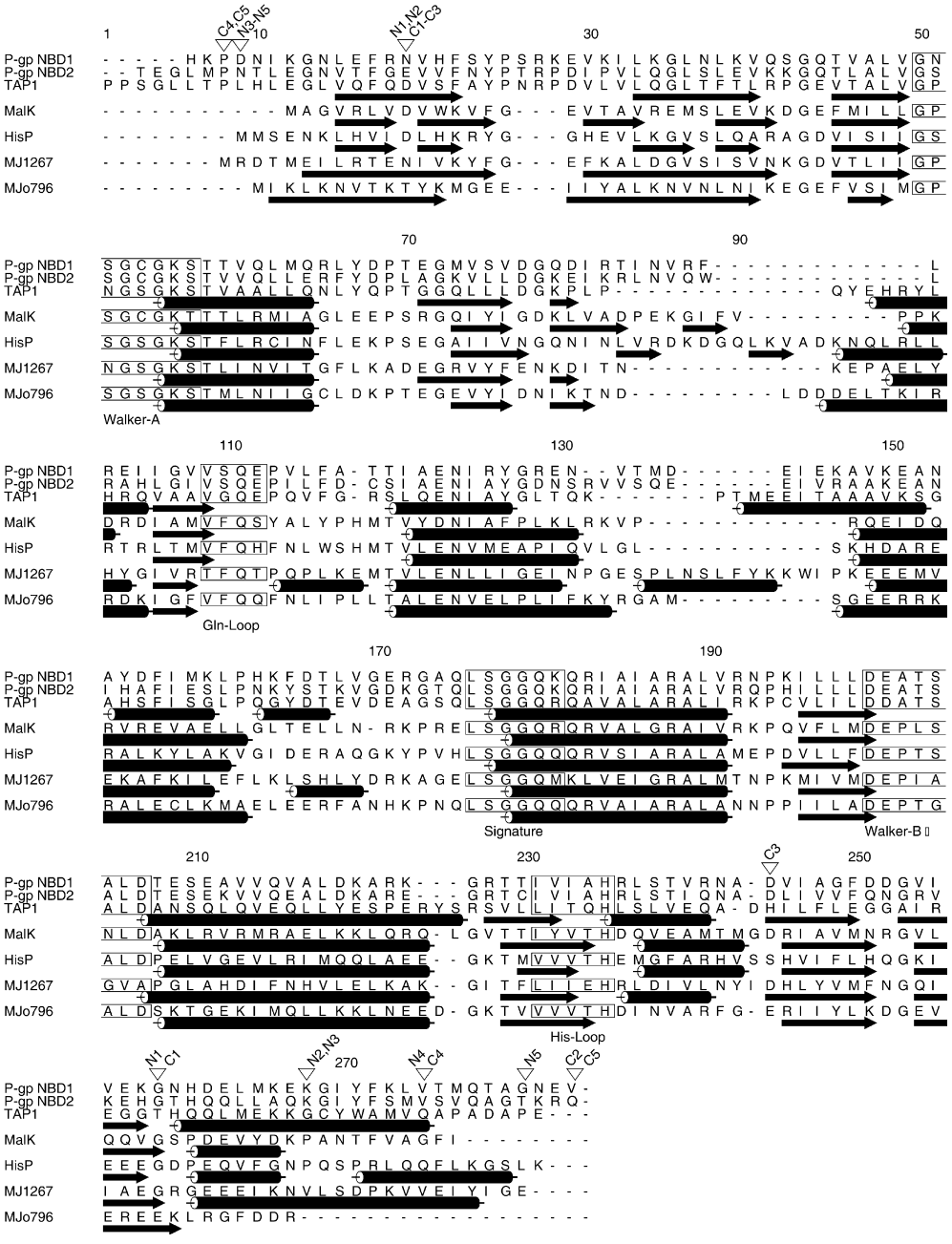

Fig. 1 Sequence alignment of ABC transporter NBDs. Sequences of ABC transporter NBDs were aligned using ClustalW (Thompson et al. 1994) and manually refined using Jalview (http://www2.ebi.ac.uk/~michele/jalview/). The resultant alignment, viewed with the program Alscript (Barton 1993), is annotated such that for each NBD whose structure has been determined the secondary structural elements are indicates by *cylinders* (α-helices) and *arrows* (*β*-strands) below that particular sequence. *Downward pointing triangles* are employed to indicate the cut-off positions of the expressed domains of P-glycoprotein NBD-N1 to NBD-N5 and NBD-C1 to NBD-C5. The five conserved sequence motifs common to all ABC proteins are *boxed* and indicated below the alignment

protease inhibitors (1 μM pepstatin A, 20 μM leupeptin, 1 mM benzamidine) and lysed by repeated 10-s bursts of sonication on ice (Misonix Microson sonicator). Cell lysis was verified by microscopic examination. Cell lysates were clarified by centrifugation at 12,000–14,000×*g* in a JA-20 rotor for 15 min at 4 °C. The supernatant from this centrifugation was centrifuged at 60,000×*g* in a TLA-100 rotor for 60 min at 4 °C. The supernatant from this second spin, containing soluble proteins, was diluted to 3 mg/mL concentration. Empirically, we determined that parallel small-scale purifications produced a higher protein yield than large-scale purifications (data not shown). Therefore, 1.5 mg aliquots of soluble protein were bound to 50 μL aliquots of Ni-NTA resin (Calbiochem, Nottingham, UK) that had been pre-equilibrated in purification buffer (150 mM NaCl, 10 mM Tris, 20% v/v glycerol, pH 6.8) with protease inhibitors (as above). Protein was allowed to bind to the resin for 30 min at room temperature with end-over-end rotation. Resin was applied to Bio-spin columns (BioRad, Hemel Hempstead, UK) and unbound proteins removed by gravity flow. Non-specific proteins were eluted with 4×2 mL washes with purification buffer supplemented with 20 mM imidazole. Specifically bound proteins were eluted by 3×2 mL washes with purification buffer containing 120 mM imidazole. Fractions were assayed for the presence of NBD proteins by SDS-PAGE or Western blotting, employing the monoclonal C219 antibody (CIS, Gif-sur-Yvette, France) as previously described (Taylor et al. 2001), and pooled. NBD-containing fractions from 25–35 parallel Ni-NTA purifications were concentrated to a final volume of ca. 1 mL under a stream of nitrogen in an Amicon stirred-cell equipped with PM10 filters (10 kDa cut-off). Concentrated Ni-NTA pure NBD protein was purified to homogeneity by gel filtration through G75 or G100 Sephadex columns (8 cm height, 3 cm radius). Fractions were assayed for the presence of NBD, pooled and concentrated as described previously.

Table 1 Oligonucleotides employed in PCR amplification of NBDs[a]

Primer	Residue	Sequence	NBD
1F	Asn396	GGAAATTTGGAAcatatgAATGTTCACTTC	N1, N2
2F	Asp386	AAGAGTGGGCACcatatgGATAATATTAAG	N3, N4, N5
3F	Glu1039	GGAAATGTCACAcatatgGAAGTTGTATTC	C1, C2, C3
4F	Pro1028	CAGCACGGAAGGCcatatgCCGAACACATTGG	C4, C5
1R	Gly610	CATGAGTTCATCctcgagTCCTTTCTCCAC	N1
2R	Lys619	AAGTTTGAAGTActcgagTTTCTCTTTCAT	N2, N3
3R	Val626	TCCTGCTGTCTGctcgagGACAAGTTTGAAG	N4
4R	Gly632	TTCTAATTCAACctcgagTCCTGCTGTCTGC	N5
5R	Gly1255	TGCCAGCAGCTGCgcggccgcGCCATGCTCCTTG	C1
6R	Gln1280	TCAGTGGTGATGCgcggccgcCTGGCGCTTTGT	C2, C5
7R	Asp1241	GTTCTGAAACACCgcggccgcGTCTGCATTCTG	C3
8R	Val1271	CTTTGTTCCAGCCgcggccgcGACCATTGAAAA	C4

[a]Forward primers carry the notation "F" and the column headed "Residue" then denotes the *initial* amino acid in the resultant NBD. Reverse primers carry the notation "R" and the column headed "Residue" then denotes the *final* amino acid in the resultant NBD. In lowercase are the restriction sites used to introduce the PCR products into pET-20. The "NBD" column indicates which NBDs were amplified using the relevant primer. As an example, the forward primer for NBD-N5 is 2F, which encodes the restriction site catatg (*Nde*I) and the reverse primer is 4R, including the restriction site ctcgag (*Xho*I). Combination of these primers enables the amplification of residues Asp386 to Gly632 of cysteine-less P-gp. All oligonucleotide sequences are given $5' \rightarrow 3'$ direction

Protein assay

Protein concentrations of bacterial lysates were determined by the DC protein assay kit (BioRad) according to the manufacturer's instructions. Since imidazole interferes with this and other commercially available protein assay kits, purified NBD protein was routinely quantified by densitometric analysis of SDS-PAGE gels. Bovine serum albumin (BSA; 0.05–1.0 μg) was loaded on SDS-PAGE gels along with triplicate aliquots of purified protein and the gels stained with Coomassie brilliant blue R250. Following destaining, gels were scanned and the intensity of the BSA bands quantified by densitometry (employing the freely available Scion Image program, www.scioncorp.com) to produce a standard curve from which the concentration of NBDs was calculated.

8-Azido-ATP labelling

Aliquots (10 μg) of NBD-N5 in a total volume of 70 µL of lysis buffer (the 20% glycerol in this buffer scavenges non-specific photo-induced free radical species) were incubated at 37 °C with [α-^{32}P]-8-azido ATP (3 µM, ICN Biochemicals, Basingstoke, UK) and increasing concentrations of unlabelled nucleotide for 10 min. Samples were returned to ice and subjected to UV irradiation (265 nm, 100 W) at a distance of 5 cm from the source for 5 min to enable photo cross-linking. Samples were precipitated by trichloroacetic acid and separated by SDS-PAGE. Photolabelled NBD-N5 was then detected by autoradiography at −80 °C and bands quantified using Scion Image. Data were analysed using Prism (GraphPad Software). The highest intensity band was designated as 100% and other data points were quantified with respect to this signal. Plots of nucleotide concentration against percent intensity were obtained and the sigmoidal dose–response curve was fitted by non-linear regression to the equation:

$$y = \text{bottom} + \frac{(\text{top} - \text{bottom})}{1 + 10^{(\log \text{IC}_{50} - \log x)^n}} \quad (1)$$

where y = percentage of reference signal, x = nucleotide concentration, bottom = minimum labelling intensity, top = saturation of labelling, IC_{50} = concentration required to reduce labelling intensity to 50% of its maximum, and n = Hill number. Data were fitted either with a Hill number of 1.0, or with a variable Hill number. An F-test was used to determine whether the data were best fit by an equation containing a fixed or variable slope.

Circular dichroism spectroscopy

NBD-N5 was dialysed into 500 mM NaCl, 50 mM phosphate buffer (pH 6.8) to avoid absorbance artifacts due to buffer components such as Tris. The circular dichroism (CD) spectra for NBD-N5 were recorded between 190 and 250 nm at 20 °C in a 0.1-cm pathlength cuvette using a Jasco J720 spectropolarimeter. Protein concentration was determined subsequent to data collection by quantitative amino acid analysis (Applied Biosystems 420A amino acid analyzer).

Sequence alignments and homology modelling

Sequences obtained from the Sequence Retrieval Server (SRS) were aligned using the package ClustalW (Thompson et al. 1994), and displayed using the program Alscript (Barton 1993). Homology models for NBD-N1 to N5 and NBD-C1 to C5 were generated using the program Modeller (v6.2, Sali and Blundell 1993), running on a 2 GHz Pentium computer. A pairwise sequence alignment extracted from the multiple sequence alignment derived above was used to produce homology models, based upon the crystal structure of TAP1 (Gaudet and Wiley 2001). A default modelling routine was employed with no further refinement of loop regions. Ten independent models for each NBD were generated. Models were analysed by comparison against a database derived from analysis of high-resolution protein structures using the What Check package (Hooft et al. 1996). Z-values calculated by What Check analysis were obtained for each model, and the mean and standard error of the mean (s.e.m.) analysed by one-way ANOVA (analysis of variance) employing the Newman–Keuls post-hoc test to determine whether there were significant differences between ensembles of models. Statistical analysis was performed using GraphPad Prism.

Results

Definition of nucleotide binding domain boundary

Sequence alignments were employed to determine the domain boundaries of the N- and C-terminal NBDs of P-gp. All domains included the five conserved NBD sequences, namely the ATP-binding motifs (Walker-A and Walker-B; Walker et al. 1982), the ABC-signature

sequence, the glutamine loop (Gln-loop; Karpowich et al. 2001) and the histidine-loop (His-loop; Linton and Higgins 1998). NBD-N1 (residues 396–610) and NBD-C1 (residues 1039–1255) define core domains with boundaries determined on the basis of residue conservation from sequence alignment (Fig. 1). A series of eight further NBDs were designed to test the requirement for secondary structural elements observed in crystallographic studies of bacterial and eukaryotic NBDs. For example, NBD-N2 investigates the effect of including residues that form a short C-terminal α-helix in MalK and HisP, while NBD-N4 and NBD-N5 make further extensions to this region, which is still α-helical in TAP1 and MJ1267 (Fig. 1). Comparable decisions were made to define alternative definitions of the C-terminal NBD. In this case, a truncated domain (NBD-C3) was also defined to investigate the requirement for the final β-hairpin in the ABC domain structure. Table 1 lists the oligonucleotide primers employed to amplify these sequences from DNA encoding cysteine-less P-gp. The use of cysteine-less P-gp avoids problems associated with expression of multiple cysteine-containing proteins in *E. coli*, the C-terminal NBD containing three endogenous cysteine residues. Cysteine-less P-gp, in which all seven cysteine residues have been replaced by serine, has identical kinetic properties in terms of nucleotide binding and hydrolysis as wild-type P-gp (Taylor et al. 2001).

Expression of NBDs is associated with inclusion body formation

pET20-NBD-N1 to pET20-NBD-C5 were expressed in the bacterial strains BL21.λDE3, BL21.λDE3.pLysS (Studier et al. 1990) or GJ1158 (Bhandari and Gowrishankar 1997). In BL21 derivatives, expression of T7 RNA polymerase is under the control of the *lac*UV5 promoter, whereas in GJ1158, expression is under control of the *pro*U promoter. Induction of NBD expression by addition of IPTG (BL21.λDE3) or sodium chloride (GJ1158) at 37 °C was associated with formation of inclusion bodies. Following cell lysis and low-speed centrifugation (12,000–15,000×*g*), the pellet was resuspended in lysis buffer supplemented with 1.0% v/v Triton X-100 and 0.5 M NaCl and re-centrifuged (12000×*g*, 10 min). Two cycles of this process resulted in the production of a Triton-insoluble pellet which was over 90% NBD when analysed by SDS-PAGE (data not shown). Although a rich source of NBDs, refolding was not pursued due to difficulties in ensuring a homogeneous population of refolded protein. As inclusion body formation is believed to be partly due to the overly rapid expression of protein, multiple attempts at reducing the problem were made. These included varying the concentration of the inducing agent (0.01–0.3 M NaCl in the case of GJ1158, and 0.05–1.0 mM IPTG for BL21.λDE3), and the temperature of bacterial culture following induction (varied from 20 °C to 37 °C). Additional experiments were carried out in which NBDs were co-expressed with chaperone proteins encoded on compatible plasmids co-transformed with pET-20 derivatives (Nishihara et al. 1998). Again, NBDs were expressed in the form of inclusion bodies, which were pelleted in the 12,000×*g* centrifugation step following bacterial lysis. An alternative strategy involved expression of NBDs together with bacteriocin release protein (BRP), which permits the release of colicins into the culture media by permeabilizing the bacterial cytoplasmic membrane (Yu and San 1992). Co-expression of NBDs with the 28 amino acid BRP was associated with release of ca. 10% of the expressed NBD into the periplasm or growth media. However, the majority of this protein was found to be in the pellet following centrifugation at 60,000×*g*, and attempts at concentrating the remaining soluble material resulted in the formation of insoluble aggregates.

Expression and purification of soluble NBD-N5

The primary goal of this research was to obtain soluble NBDs for P-gp which may be suitable for biochemical and structural analysis. Thus, it is imperative to produce protein which remains in the supernatant subsequent to centrifugation at 60,000×*g*. Since expression under the control of the *lac* operon failed this requirement, all 10 NBDs were subsequently sub-cloned from pET-20 into a vector derived from pBAD-GFP*uv* in which protein expression is placed under the control of the *araBAD* operon. NBD-N5 was expressed as a soluble protein (i.e. was located in the supernatant following centrifugation for 60 min at 60,000×*g*) in GJ1158 or BL21 strains of *E. coli* by incubation of bacterial cultures at 18–22°C following induction by 0.02% L-arabinose. Despite extensive attempts employing diverse growth temperatures and concentrations of L-arabinose, only NBD-N5 was expressed in the cytoplasm as a soluble protein under the control of the *araBAD* operon. All other NBDs were found to be associated with insoluble inclusion body deposits (data not shown).

NBD-N5 was purified from *E. coli* in two stages. The first step (Fig. 2A) employed immobilized metal affinity chromatography. Incubation of soluble proteins with Ni-NTA resin results in the binding of at least 75% of NBD-N5, as judged by Western blotting. Successive washing of the resin with a low concentration of imidazole (20 mM) results in the removal of most contaminating bands (lanes W1 and W2). NBD-N5 (ca. 30 kDa) and other Ni-NTA binding proteins are eluted by successive incubation of the resin with 120 mM imidazole. Attempts to avoid the co-purification of the contaminating band at ca. 70 kDa (Fig. 2A) were made by eluting from the column with a step gradient of imidazole. However, the majority of NBD-N5 and the higher molecular weight band were always present in the same fractions. Therefore, following affinity chromatography, the contaminating higher molecular weight protein (which Western blotting with monoclonal antibody C219 revealed was not related to NBDs) was removed by gel filtration

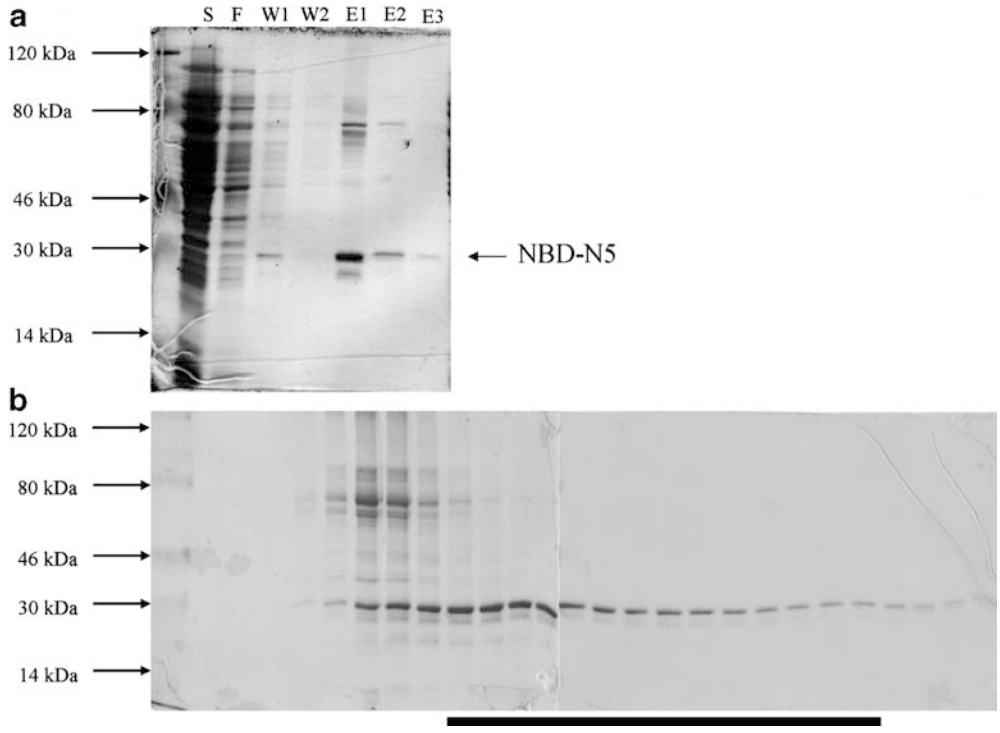

Fig. 2a, b Purification of NBD-N5. **a** Ni-NTA affinity chromatography of NBD-N5 was carried out as described in Materials and methods. Aliquots of each fraction (50 μL) were precipitated by TCA, prior to electrophoresis through 12% polyacrylamide gels, which were subsequently silver-stained. Lanes shown represent soluble proteins (*S*), unbound proteins (*F*), two wash fractions (*W1* and *W2*), and three elution fractions (*E1–E3*). *Arrows* represent the positions of molecular weight markers. **b** Gel filtration of NBD-N5. Successive fractions from a Sephadex-G75 column are displayed following silver staining. Twenty-eight 2 mL fractions were collected and 100 μL of each fraction was TCA precipitated and separated on a 12% acrylamide gel. The *arrows* represent the positions of the molecular weight markers. The figure is a composite of two gels representing fractions 1–14 and 15–28. The *solid black bar* indicates the fractions pooled and concentrated

chromatography (Fig. 2B), resulting in the production of NBD-N5, judged by densitometric analysis of SDS-PAGE gels to be >95% pure. Typically, under these culture and expression conditions, a 400 mL culture yielded 50 mg of total soluble protein, 12 mg of which was eluted from Ni-NTA resin. Following gel filtration and concentration of NBD-N5 containing fractions, 0.5–1.0 mg of NBD-N5 at a concentration of 0.2 mg/mL could be obtained. This yield is comparable to a recent study employing a similar expression system to isolate the N-terminal NBD of the cystic fibrosis transmembrane conductance regulator (CFTR; Duffieux et al. 2000). Attempts to concentrate further NBD-N5 led to the formation of aggregated species.

NBD-N5 adopts a defined secondary structure

Having produced soluble NBD-N5, the secondary structure of the domain was investigated using CD spectroscopy. Figure 3 shows the averaged spectrum of NBD-N5 subsequent to dialysis into phosphate buffer, thus removing any trace of Tris and imidazole which has significant absorbance in the UV region. The spectrum shows characteristics of a folded protein with double minima at 209 and 220 nm. Evaluation of the spectra using the program Dicroplot (Deleage and Geourjon 1993) resulted in a calculated α-helical content of 35%, which is consistent with the high-resolution structure of TAP1 (Gaudet and Wiley 2001) and prokaryotic NBD proteins (averaged secondary structure content of TAP1, HisP and MalK is 38% α-helix, 22% β-sheet and 40% other structures), further demonstrating that NBD-N5 has adopted a stable tertiary structure.

NBD-N5 displays specific nucleotide binding

In the previous section the soluble NBD-N5 was demonstrated to have secondary structure comparable with

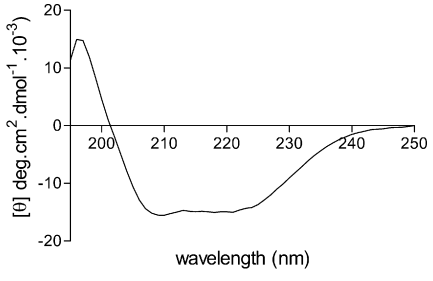

Fig. 3 Circular dichroism spectroscopy of NBD-N5. CD spectra were obtained in a 1-mm pathlength cuvette at 20 °C at a protein concentration of 2.4 μM in a 50 mM phosphate buffer (pH 6.8) containing 500 mM NaCl. The molar ellipticity is plotted as a function of wavelength and represents the average of 10 scans

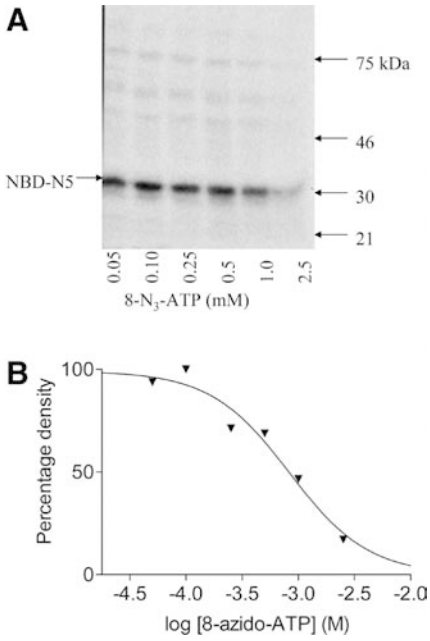

Fig. 4A, B Interaction of NBD-N5 with nucleotide. **A** Binding of [^{32}P]-8-azido-ATP was performed as described in the text, with increasing concentrations (as marked) of unlabelled 8-azido-ATP as competing ligand. Following 5 min irradiation on ice, proteins were separated by 15% SDS-PAGE and detected by autoradiography at −80 °C. The positions of molecular weight markers are indicated at the *right-hand side*. **B** The sigmoidal dose–response curve fit to the data shown in **A** is presented, with a calculated IC_{50} of labelling of 0.9 mM

NBDs that have been resolved at high resolution. To augment these data, functional characterization of NBD-N5 by ATPase activity was performed. However, under the reactions conditions tested, NBD-N5 displayed no ATPase activity according to a colorimetric phosphate release assay (Chifflet et al. 1988), which can detect as little as 1 nmol of released inorganic phosphate. Neither could ATPase activity be detected with other commercially available assays. In the absence of ATPase activity it was of importance to determine specific nucleotide binding by NBD-N5 to provide supporting evidence that it is an ordered domain in the absence of cognate TMDs. Figure 4 displays the results of [α-^{32}P]-8-azido-ATP binding assays with NBD-N5. The maximum concentration of this nucleotide commercially available is 100 μM in methanol. The need to avoid excess solvent in labelling experiments thus imposes an upper concentration limit of ca. 5 μM. This precludes a direct measurement of the Michaelis constant, K_m [which is in the region of 0.5 mM in intact P-gp (Taylor et al. 2001)]. This low affinity also precludes radioligand binding assays with high specific activity [^{32}P]ATP. Therefore, we have performed assays in which NBD-N5 was incubated with increasing concentrations of unlabelled nucleotide to prevent cross-linking of protein with [α-^{32}P]-8-azido-ATP. Figure 4A is an autoradiogram demonstrating that [α-^{32}P]-8-azido-ATP labelling is completely prevented by increasing the concentration of unlabelled 8-azido-ATP to 2.5 mM. Figure 4B is the sigmoidal dose–response curve fit to the data, from which a value of 0.9 mM was obtained for the half-maximal inhibition of [α-^{32}P]-8-azido-ATP labelling by 8-azido-ATP (IC_{50}). Analysis of data from three independent experiments produced a mean EC_{50} value of 1.20 mM (s.e.m. 0.18 mM). Similar results were obtained if the competing nucleotide was ATP, although the potency was reduced to an IC_{50} of 2.8 mM, demonstrating that NBD-N5 has the ability to specifically bind nucleotide.

NBD-N5 is monomeric in solution

Recent data suggest that the NBDs of ABC transporters are tightly associated in the intact transporter (Locher et al. 2002), and that this association may be triggered by the binding of ATP (Fetsch and Davidson 2002; Hopfner et al. 2000; Smith et al. 2002). In order to investigate whether this might be the case for P-gp, we performed size exclusion chromatography on NBD-N5 in the presence and absence of 1 mM ATP. In both cases, NBD-N5 eluted from the column as a single peak, with an elution time consistent with a monomeric species (data not shown). The failure to show ATP-dependent dimerization of NBD-N5 may be related to the fact that the intact transporter would contain a heterodimeric NBD. Thus, the non-identical sequences of N- and C-terminal NBDs may underlie a specific, stable heterodimerization.

Discussion

Heterologous expression of P-gp NBDs

In this work we have investigated expression of the NBDs of human P-gp in a heterologous system. Related investigations on prokaryotic ABC transporters, which support the study of isolated domains from ABC transporters, have proved difficult to extend to eukaryotic systems due the high probability of inclusion body formation upon over-expression of NBDs (Booth et al. 2000; Dayan et al. 1997). We too have encountered considerable problems in the expression of soluble P-gp NBD proteins in prokaryotic cells. We do not regard the refolding of inclusion body material as being an effective means to produce recombinant eukaryotic NBDs for two reasons. Firstly, the homogeneity of the refolded species may be difficult to guarantee and would require validation by, for example, NMR spectroscopy. Secondly, the specific activity (or nucleotide binding affinity) of refolded protein must be compared with soluble protein that has not undergone a harsh denaturation and renaturation protocol (Rudolph et al. 1997). The absence of a high ATPase activity for isolated eukaryotic NBDs precludes this. Therefore, we hypothesized that the production of soluble NBDs would be best

effected by optimization of the expression system and judicious design of the expressed domain. This has enabled us to express a soluble N-terminal NBD from P-gp. A similar expression approach has recently demonstrated expression of the N-terminal NBD from CFTR (Duffieux et al. 2000). Both of these successful attempts have relied on the use of pBAD-derived plasmids in which protein expression is under the control of the *araBAD* promoter (Guzman et al. 1995).

Characterization of a soluble N-terminal NBD for P-gp

As discussed above, we have managed to express soluble protein corresponding to the N-terminal NBD of P-gp. In this study we have been unable to demonstrate ATP hydrolysis by NBD-N5 despite employing numerous assays. This is in agreement with other studies which have reported very low or undetectable levels of ATP hydrolysis by P-gp NBDs (Booth et al. 2000; Sharma and Rose 1995; Wilkes et al. 2002). We believe that full ATPase function of the P-gp NBDs requires either interaction between both NBDs [as suggested previously (Sharma and Rose 1995)], or with the cognate TMDs. We have, however, demonstrated specific binding of ATP to NBD-N5 by employing the photoactivatable derivative 8-azido-ATP. Our IC_{50} values for the inhibition of [^{32}P]-8-azido-ATP labelling by nucleotide (ca. 1 mM) are comparable with those previously obtained on refolded P-gp N-terminal NBD (Booth et al. 2000) and with those in a study on a very short form of the mouse P-gp N-terminal NBD (Dayan et al. 1996). In addition, the IC_{50} values compare well with the affinity of full-length P-gp for azido-ATP and ATP (Urbatsch et al. 1994).

Soluble NBD expression is critically dependent on the domain boundary definition

In this paper we have demonstrated that the domain boundaries chosen for the NBDs are critical to their expression in a soluble form. In this respect our data can be compared to that of other investigations (Booth et al. 2000; Dayan et al. 1996; de Wet et al. 2001). For example, our refinement of domain boundaries has identified residues 386–632 as being a stable, functional protein. This is slightly shorter than that obtained in a recent study by Pastan and colleagues (Booth et al. 2000), which identified residues 375–635 as being the functional domain boundaries. However, the primary difference in our approach is that NBD-N5 is produced in a soluble form in the bacterial cytoplasm, whereas the longer polypeptide required refolding after isolation of inclusion bodies (Booth et al. 2000). Similarly, Di Pietro and colleagues expressed recombinant polypeptides of 187, 219, 249 and 336 amino acids corresponding to the N-terminal NBD of mouse P-gp (Dayan et al. 1996, 1997). Only the shortest was found to be soluble, the

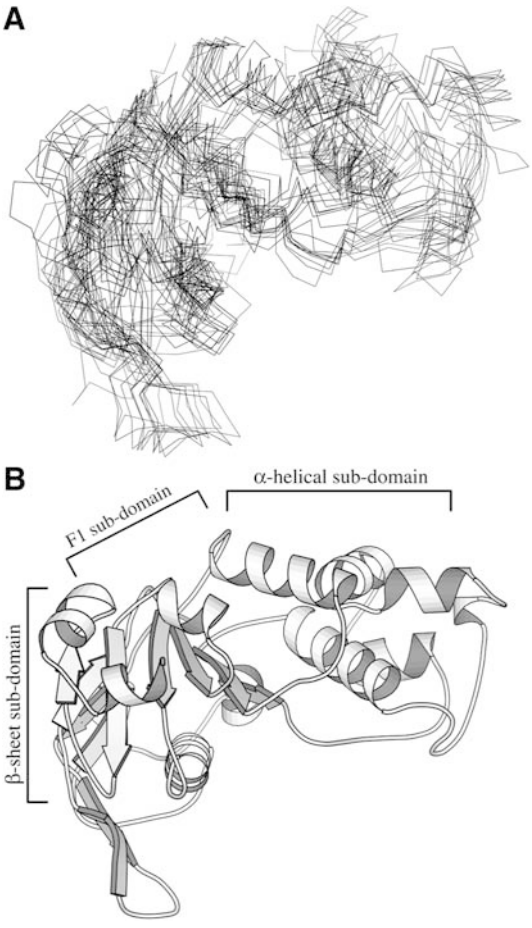

Fig. 5A, B Structural modelling of NBD-N5. **A** For each NBD an ensemble of 10 models was produced using Modeller. The entire ensemble of NBD-N5 models superposed by least squares alignment of their Cα atoms is shown in the form of a *chain trace*. **B** A representative of the NBD-N5 ensemble is shown with secondary structural elements represented by *ribbons* (α-helices) and *arrows* (β-strands). The figure was produced using Molscript (Kraulis 1991)

three longer polypeptides being associated with inclusion bodies. Additionally, the same group has investigated different lengths of polypeptide to define the C-terminal NBD of mouse P-gp (de Wet et al. 2001), although in these cases the presence of detergents was present in the protein purification buffers, which may aid protein solubility. Interestingly, our domain definition (for NBD-N5) is almost exactly the same as that employed to produce TAP1-NBD protein for crystallographic studies (Gaudet and Wiley 2001), NBD-N5 being five amino acids shorter at the 5′-end.

We can now interpret our original sequence alignment to provide plausible explanations for the failure of nine of our NBDs to form soluble proteins. NBD-N1, NBD-N2 and NBD-C1 to NBD-C3 lack one-strand of an N-terminal β-sheet found in NBDs that have been structurally characterized at high resolution (Fig. 1). The presence of this β-sheet is clearly a requisite for the stability of the NBD, although its sequence is not highly conserved (Kerr 2002). The remaining five NBDs have a

Table 2 Structural quality of homology models of N- and C-terminal NBDs[a]

	N1	N2	N3	N4	N5	C1	C2	C3	C4	C5
$\chi_1\chi 2$	−0.04 (0.16)	−0.14 (0.13)	−0.28 (0.12)	−0.01 (0.14)	−0.04 (0.08)	0.33 (0.16)	0.14 (0.14)	−0.38 (0.21)	−0.01 (0.17)	−0.01 (0.17)
$\phi\psi$	1.14 (0.07)	1.02 (0.07)	1.13 (0.06)	1.36* (0.07)	1.30* (0.07)	0.82 (0.06)	0.94 (0.07)	0.73 (0.07)	1.04 (0.04)	0.89 (0.07)

[a]Z-values (reported by the What Check package) are given as the mean (s.e.m.); $n = 10$ in all cases. The secondary structural quality analysis is denoted by "$\phi\psi$", the symbols assigned to the backbone torsion angles. The rotamer quality analysis is denoted by "$\chi_1\chi 2$", the symbols assigned to the sidechain torsion angles. Mean values of ensembles of models that are significantly different to all other ensembles of models are indicated by an asterisk ($P < 0.05$). Higher *Z*-values indicate better structural quality

common N-terminal definition, suggesting that reasons for the failure of these domains to be expressed in a soluble form must involve the C-terminus. The failure of NBD-N3, NBD-N4 and NBD-C4 to express in a soluble form suggests that the final α-helix in the NBD, although poorly conserved in sequence, is a critical structural element.

In order to provide further evidence that exact definition of domain boundary constraints is essential to producing folded soluble protein, we have produced homology models of the 10 NBD definitions. For each NBD, an ensemble of 10 independent models was generated, employing as a template the crystal structure of TAP1 (Gaudet and Wiley 2001), which displays the highest end-to-end sequence identity with the NBDs of P-gp. Each ensemble (Fig. 5A) was visually inspected to ensure that the alignment had not introduced any significant distortions into the expected L-shape structure of an ABC transporter NBD (Hung et al. 1998) (Fig. 5B). Models were analysed for two criteria, namely the stereochemical quality of the side-chains and the stereochemical quality of the backbone. The former is a comparison against backbone rotamer libraries, while the latter is a measure of the percentage of amino acids whose backbone torsion angles occupy energetically favourable regions of the Ramachandran plot. The two criteria are assigned a score (higher *Z*-value suggesting an energetically more favourable structure) which was tabulated for each model. The mean data, which therefore indicates the quality of the entire ensemble for these two characteristics, is shown in Table 2. For the sidechain rotamer criterion, ANOVA demonstrated that the differences in the mean values are likely to be observed by chance ($P > 0.05$). Therefore, sidechain conformations do not provide any hypothesis for the successful expression of NBD-N5. In contrast, ANOVA demonstrated that the difference in the mean *Z*-score for secondary structure was unlikely to be observed by chance ($P < 0.001$). Post-hoc comparison of the mean *Z*-scores for individual ensembles demonstrates that NBD-N4 and NBD-N5 have more favourable secondary structure ($P < 0.05$) than all other ensembles produced. No other parameter analysed by What Check was able to rationalize the expression of NBD-N5 as a soluble protein and the expression of NBD-N4 as an inclusion body bound protein. The less favourable backbone conformation of the C-terminal NBDs suggests that the C-terminal domain in isolation may be less stable, leading to its deposition in inclusion bodies. This suggests a reason why NBD-N5 was expressed as a soluble domain, while NBD-C5 was not. The requirement for detergents in the extraction of a C-terminal P-gp NBD previously reported is consistent with this (de Wet et al. 2001).

In summary, we have undertaken a detailed investigation of the expression of NBDs from P-glycoprotein as soluble proteins in *E. coli*. We have determined that the expression of such proteins is fraught with difficulties relating to the production of inclusion bodies. However, we have shown that a combination of judicial choice of domain boundaries and refinement of expression conditions can enable production of soluble protein, which is suitable for structural studies. To our knowledge, this is the first demonstration that the N-terminal NBD of human P-gp can be expressed and remain in the soluble fraction following centrifugation at 60,000×*g*. The strategy we have adopted involving detailed analysis of sequence alignments and homology modelling may have applications for expressing domains of other multi-domain membrane proteins.

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