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Purification of functional human P-glycoprotein expressed in *Saccharomyces cerevisiae*

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Abstract

A system for expression and facile purification of the human P-glycoprotein (Pgp) from the yeast *Saccharomyces cerevisiae* is described. The wild-type human *mdr1* cDNA was cloned into a high copy number yeast expression vector under the control of the constitutive promoter of the yeast plasma membrane H^+-ATPase. Western blots of membranes from the stable transformants confirmed that the Pgp is expressed in yeast cells in amounts approximately 0.4% of the total yeast membrane protein. Density gradient sedimentation analysis of the yeast membranes indicated that the expressed Pgp is localized in the plasma membrane. Yeast cells transformed with the Pgp expression plasmid acquire increased resistance to valinomycin, suggesting that the expressed Pgp is properly folded and functional. The expressed Pgp can be solubilized from the yeast membranes with lysophosphatidylcholine, and when tagged with ten histidines at its C-terminus, can be readily purified to about 90% homogeneity by Ni^{2+} affinity chromatography. About 50 μg of the Pgp can be purified from 20 mg of crude yeast membranes. The purified human Pgp exhibits a verapamil-stimulated ATPase activity and the maximal activity is 2.5 ± 0.5 μmol/min per mg of Pgp, suggesting that the purified Pgp from yeast is highly functional. The Pgp expressed in yeast has the same electrophoretic mobility (ca. 130 kDa) as the Pgp produced in Sf9 insect cells and is unaffected by *N*-glycosidase treatment, suggesting that it is not glycosylated. Because of the relative ease of growing yeast in massive quantities this expression system appears to be excellent for producing this membrane transporter at levels sufficient for further biochemical and biophysical studies, and for site-directed mutagenesis studies as well. © 1997 Elsevier Science B.V.

Keywords: Expression; Purification; P-glycoprotein; Glycoprotein; Human Pgp; Yeast

1. Introduction

The development of multidrug resistance (MDR) in cancer is a significant obstacle to the successful chemotherapy of cancer. A major form of MDR is caused by the over-expression of a 170 kDa membrane protein known as the P-glycoprotein (Pgp). The Pgp is generally thought to pump a variety of structurally and functionally unrelated hydrophobic antitumor agents out of resistant tumor cells, thus sparing

Abbreviations: MDR, multidrug resistance; CFTR, the human cystic fibrosis transmembrane conductance regulator; Pgp, P-glycoprotein; EDTA, ethylenediaminetetraacetic acid dipotassium salt dihydrate; PMSF, phenylmethanesulfonyl fluoride; PCR, polymerase chain reaction; LPC, L-α-lysophosphatidylcholine; DDM, dodecyl-β-D-maltopyranoside; ABC, ATP binding cassette; NTA, nitrilotriacetic acid

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them from the killing action of these drugs [1–4], although alternative explanations of the available data have been proposed [5]. Human Pgp is encoded by the *mdr1* gene, and shares significant sequence homology with several bacterial transporters [6] and with mammalian proteins such as the cystic fibrosis transmembrane conductance regulator (CFTR). It belongs to the ATP-binding cassette (ABC) or traffic-ATPase superfamily of transport proteins [7,8]. Although extensive biochemical studies have been carried out with the Pgp, much remains to be learned about its structure and molecular mechanism of drug transport. Future efforts to understand in more detail the structure and function of the Pgp will require large quantities of purified functional protein. Since the endogenous levels of the human Pgp in natural sources are low, suitable starting materials for Pgp purification can only be obtained from overproducing cultured cell lines or high-yield heterologous expression systems. Excellent Pgp production and purification systems using cultured drug-resistant cell lines have been recently reported [9–12]. However, major drawbacks of these systems are the expense and labor inherent in tissue culture systems and the inability to mutate the Pgp. Heterologous expression systems for the Pgp in yeast [13–15], Sf9 insect cells [16,17], *E. coli* [18,19], and transiently transfected human embryonic kidney 293 (HEK293) cells [20] have been developed in recent years. The Sf9 insect cell/baculovirus expression system appears to be a promising system for providing large amounts of starting material for biochemical studies but it involves cell culture techniques as difficult and expensive as the drug-resistant animal cell systems. Recently, human Pgp and its mutants have been purified from transiently transfected HEK293 cells but yields of purified protein are relatively low [20]. In contrast to cultured cell systems, yeast expression systems require only simple culture techniques and inexpensive media and allow the production of substantial quantities of eukaryotic proteins. Moreover, the thoroughly developed methodology and the readily manipulated techniques of yeast molecular genetics can provide novel approaches for studying the expressed proteins, which is more difficult in mammalian and insect cell expression systems. Although the mouse and the human Pgps have been expressed in yeast [13–15], the expression levels are difficult to estimate from the data presented. In this laboratory, yeast expression systems for the *Neurospora crassa* plasma membrane H^+-ATPase [21] and the CFTR [22] have recently been developed, based on the system established by Serrano and co-workers [23]. The success of these studies prompted us to extend this expression system to the human Pgp. In the present study we report a yeast expression system that produces functional human Pgp in reasonable yield and allows for subsequent facile solubilization and purification of the Pgp to near homogeneity. The availability of this new expression system may facilitate future studies for elucidating the structure and molecular mechanism of this clinically important membrane transport protein.

2. Materials and methods

2.1. Plasmid constructions

Standard recombinant DNA techniques [24] were used to construct plasmids containing the human *mdr1* cDNA for expression in *Saccharomyces cerevisiae*. We began with the expression vector pSKMHA9, which was previously used for expression of the *Neurospora crassa* plasma membrane H^+-ATPase in yeast [25]. To remove the majority of the H^+-ATPase cDNA from the expression vector, pSKMHA9 was digested with *Nco*I and *Nde*I, treated with Klenow enzyme, and religated. The resulting plasmid was digested with *Xho*I and religated to produce plasmid pSKMHA9$N^-N^-X^-$. The wild-type human *mdr1* cDNA, taken from plasmid pHaMDRGA [26] by digestion with *Bst*UI and *Xho*I, was then ligated with the vector fragment produced from pSKMHA9$N^-N^-X^-$ by digestion with *Not*I, filling in the ends with Klenow enzyme, and digestion with *Xho*I. This construct, pMDR1, contains the coding region of the wild-type human Pgp in a yeast expression vector under the control of the constitutive promoter of the yeast plasma membrane H^+-ATPase.

*Pst*I, *Sal*I and *Sph*I sites in the polylinker of pMDR1 were then deleted by digestion of pMDR1 with *Bam*HI and *Sph*I, filling in the ends with Klenow enzyme, and religation to produce plasmid pQMMDR1 in which there is an unique *Pst*I site about 500 base pairs upstream from the stop codon of

the *mdr1* cDNA. To facilitate Pgp purification, a histidine tag containing 10 tandem histidines was introduced at the C-terminus as follows: Two primers were designed to amplify a PCR product containing the histidine tag using pQMMDR1 as template. One primer annealed between *Bcl*I and *Pst*I sites, and the other annealed to the 3′ end of the *mdr1* cDNA. The latter primer contained a DNA sequence coding for a histidine tag which was immediately followed by a TAG stop codon, and *Mlu*I and *Xho*I sites. The PCR fragment containing a histidine tag was then digested with *Pst*I and *Xho*I, and cloned into pQMMDR1 digested with the same enzymes. The resulting construct was named pQMMDR2.

Because of the observation that translation initiation may occur at an in-frame ATT upstream from the ATG start codon [22], which may cause N-terminal extension of the translated protein, the sequence containing all of three such ATTs present in pQMMDR2 were deleted by overlap extension using the polymerase chain reaction [27] and, at the same time, an unique *Sac*II site was introduced immediately preceding the ATG start codon, as shown in Fig. 1. To do this, a PCR fragment of approximately 2.3 kb was produced by overlap extension of two PCR products obtained from the first PCRs from *Sma*I and *Apa*I to mutagenic primers (see Fig. 1), and then the 2.3 kb PCR product, in which the sequence upstream from the ATG start codon is exactly the same as that in the expression plasmid pHCFTRW as described by Huang et al. [22], was digested with *Sma*I and *Apa*I, purified in low-melting agarose and ligated with the vector fragment from pQMMDR2 also digested with *Sma*I and *Apa*I. This construct, pQMMDR4, is shown in Fig. 1. It contains codons for a histidine tag attached to the C-terminus of the Pgp and has deleted all of three in-frame ATTs upstream from the ATG start codon. The same PCR fragment was also cloned into pMDR1 to produce plasmid pQMMDR3. Finally, both DNA sequences within the *mdr1* cDNA (from *Sac*II to *Apa*I, and from *Pst*I to *Xho*I) generated by PCR were completely sequenced at the UNC-CH Automated DNA Sequencing Facility on a Model 373A DNA Sequencer (Applied Biosystems) using the Taq DyeDeoxy™ Terminator Cycle Sequencing Kit (Applied Biosystems) before yeast transformation to confirm that no errors had been introduced.

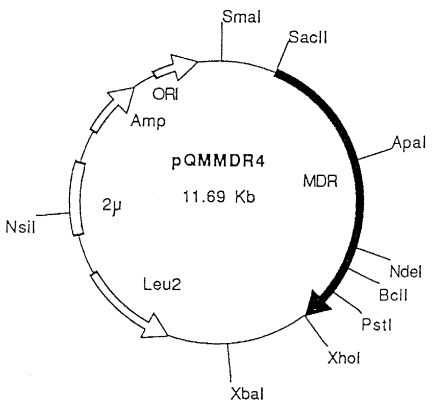

Fig. 1. Plasmid map of pQMMDR4. Plasmid pQMMDR4 encodes the human Pgp under the control of the constitutive promoter of the yeast plasma membrane H^+-ATPase. Codons for a histidine tag at the C-terminus of the Pgp are also included. The filled box represents the cDNA of the Pgp, and the arrow of the filled box indicates the sense direction of the cDNA. Some important restriction sites used for cloning are indicated as well as several important genetic markers.

2.2. Strain and media

S. cerevisiae proteinase-deficient strain BJ1991 (*α leu2 trp1 ura3-52 prb1-1122 pep4-3 gal2*) was used for transformation with the *leu2* plasmids throughout this work. Yeast clones transformed with *leu2* plasmids were grown on synthetic medium containing 0.17% (w/v) yeast nitrogen base without amino acids, 0.5% (w/v) ammonium sulfate, 2% (w/v) D-glucose, 0.2 mM adenine, and 0.2% (w/v) of an amino acid mixture lacking leucine (SC-LEU, Bio101, La Jolla, CA). Untransformed yeast cultures were grown in the same medium but supplemented with 1 mM L-leucine. Solid media additionally contained 1.5% (w/v) Difco agar and, in certain cases, 150 μM valinomycin (Sigma) added from ethanol solution.

2.3. Yeast transformation and growth tests

Transformation of yeast cells was carried out as described previously [21]. Transformation of yeast strain BJ1991 with plasmids pQMMDR3 and pQMMDR4 gave rise to yeast strains QMMDR3 and QMMDR4. Transformation with the control plasmid pRS421-Not [21], which does not contain the *mdr1*

cDNA, gave rise to strain RS421-Not. For growth rate experiments, cells were first grown in synthetic medium to an A_{600nm} of about 1. Subsequently, for the liquid growth assay, cells were diluted in the same medium to $A_{600nm} = 0.015$. The diluted cells were then grown with constant agitation (140 rpm) at 30°C, and growth was monitored by measuring the A_{600nm}. For the quantitative analysis of valinomycin resistance of Pgp-expressing yeast cells, cells were diluted to $A_{600nm} \cong 0.05$, the diluted cells were grown with constant agitation (140 rpm) at 30°C, and cell growth was scored every 2–4 h as the A_{600nm}. Valinomycin was added into the media from ethanol solution. Growth on solid media was assessed using a drop test, similar to that previously described [21]. Briefly, the Pgp and control transformants grown to $A_{600nm} \cong 1$ were diluted to the same cell concentrations, and then 10^4 cells were spotted on agar plates with or without valinomycin. Cell growth was recorded after 48 h at 30°C.

2.4. Isolation of membranes

As for the expression of the AE1 anion exchanger in yeast [28], we have observed that the slowest growing transformants express the highest level of the Pgp. Therefore, only the slowest growing transformants, which usually have a doubling time greater than 7 h, were selected for membrane preparation. Cells of strain QMMDR4 grown to $A_{600nm} \cong 1$ were harvested by centrifugation, washed with cold water and resuspended in homogenization buffer, as described previously [21,22] with minor modifications. The homogenization buffer contained 25 mM Tris, 1 mM EDTA, 1 mM PMSF, 2 μg/ml chymostatin, leupeptin, pepstatin A and aprotinin, 1 mM β-mercaptoethanol, and 10% (w/v) glycerol, pH 7.5 with HCl. Then 5–10 g of yeast cells in 20 ml of homogenization buffer were mixed with 50 g of glass beads and cooled to -5°C for 10 min in a freezer and the cells were then broken in a Braun cell homogenizer (B. Braun Biotech Inc., Allentown, PA) for 45 sec. The homogenizer chamber was kept at 4°C using liquid CO_2 during the homogenization process. Cell debris and glass beads were separated from the supernatant fluid and washed once with 10 ml of homogenization buffer/g of cells by centrifugation for 10 min at 1600 × g (4°C). Both supernatant fluids were pooled and the membranes were sedimented by centrifugation for 1 h at 200 000 × g at 4°C. The membranes were resuspended in storage buffer containing 25 mM Tris, 1 mM EDTA, 1 mM PMSF, 2 μg/ml chymostatin, leupeptin, pepstatin A and aprotinin, 1 mM β-mercaptoethanol, and 20% (w/v) glycerol, pH 7.5 with HCl. The protein concentration of the membrane suspensions was 11–13 mg/ml. The membrane suspensions were divided into aliquots and stored at -80°C until use.

2.5. Sucrose gradient centrifugation

The isolated membranes in storage buffer (3 mg of protein) were layered on the top of a discontinuous sucrose gradient containing 2 ml aliquots of 25%, 30%, 35%, 40% and 45% (w/v) sucrose in gradient buffer in a 12 ml centrifuge tube. The gradient buffer contained 10 mM Tris, 1 mM EDTA, 1 mM PMSF, 2 μg/ml chymostatin, leupeptin, pepstatin A and aprotinin, and 1 mM β-mercaptoethanol, pH 7.5 with HCl. The gradient was centrifuged at 150 000 × g for 3 h at 4°C in a Beckman SW 40 Ti rotor. Fractions (0.7 ml each) were collected from the top of the gradient and assayed for protein concentration. Then 20 μg of protein from membrane-containing fractions from the top to the bottom of the gradient was treated and analyzed essentially as described previously [22]. The Pgp was first detected using immunoblotting. After a luminogram of immunostaining for the Pgp had been obtained the same blot was washed for 1 h in TBS–Tween buffer [17] and reblotted for the yeast plasma membrane H^+-ATPase.

2.6. Solubilization and purification of the expressed recombinant human Pgp

Typically, membranes (20 mg of protein) were washed twice with buffer A (25 mM Tris, 1 mM PMSF, 400 mM NaCl, 2 μg/ml chymostatin, leupeptin, pepstatin A and aprotinin, 1 mM β-mercaptoethanol, 20 mM imidazole, and 20% (w/v) glycerol, pH 8.0 with HCl) by centrifugation (100 000 × g, 15 min, 4°C) and were then solubilized with LPC (Sigma, from egg yolk, approximately 99% pure) at a ratio of 5 mg LPC/mg membrane protein in buffer A at a protein concentration of 2 mg/ml. After incubation

with gentle stirring for 20 min at 4°C, the solution was centrifuged (100 000 × *g*, 45 min, 4°C) to remove insoluble materials. The supernatant fluid containing the solubilized Pgp was then mixed with 1 ml of Ni^{2+}-NTA resin equilibrated with buffer B (25 mM Tris, 1 mM PMSF, 400 mM NaCl, 2 μg/ml chymostatin, leupeptin, pepstatin A and aprotinin, 1 mM β-mercaptoethanol, 30 mM imidazole, 20% (w/v) glycerol, and 0.1% (w/v) DDM (Boehringer Mannheim, Mannheim, Germany), pH 8.0 with HCl) and gently shaken for 1 h at 4°C. The resin with the adsorbed protein was then packed into a 1 × 5 cm column (Bio-Rad). After the flow-through was passed and collected, the column was washed with 10 ml of buffer B and 20 ml of buffer C (25 mM Tris, 1 mM PMSF, 400 mM NaCl, 2 μg/ml chymostatin, leupeptin, pepstatin A and aprotinin, 1 mM β-mercaptoethanol, 60 mM imidazole, 20% (w/v) glycerol, and 0.1% (w/v) DDM, pH 7.5 with HCl). The Pgp was then eluted with buffer D (25 mM Tris, 1 mM PMSF, 20 mM NaCl, 2 μg/ml chymostatin, leupeptin, pepstatin A and aprotinin, 1 mM β-mercaptoethanol, 250 mM imidazole, 20% (w/v) glycerol, and 0.1% (w/v) DDM, pH 7.5 with HCl). The flow rate was set to 0.5 ml/min and 1 ml fractions were collected. Fractions were analyzed by SDS–PAGE in 7.5% (w/v) acrylamide gels, which were either stained with alkaline silver [29] or transferred to nitrocellulose membranes for immunostaining of the Pgp. The fractions containing the Pgp were pooled and assayed for protein concentration. Purified Pgp samples were concentrated to 0.3 mg/ml in a Centricon 100 (Amicon) and stored in aliquots at −20°C until use. Solubilization and purification of the Pgp were carried out at 4°C.

2.7. Pgp ATPase activity assay

Purified Pgp (150 ng) was incubated at 37°C in 0.1 ml of buffer containing 50 mM Tris, pH 7.4 with HCl, 4 mg/ml acetone/ether-precipitated *E. coli* lipids (Avanti Polar Lipids, Alabaster, AL), 5 mM $MgSO_4$, and 5 mM NaATP for 20 min. Verapamil (Sigma) was included in the assay mixtures at a variety of concentrations. Reactions were stopped by the addition of 0.1 ml of 5% (w/v) SDS and the amount of inorganic phosphate released was determined immediately, as described by Sarkadi et al. [17]. The added verapamil solution made no contribution to the color reaction in control incubations.

2.8. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis, staining and immunoblotting

SDS–PAGE and immunoblotting were carried out essentially as described [17,21,22]. Electrophoresis was usually carried out in 7.5% (w/v) SDS–PAGE minigels in a Bio-Rad Mini-Protean II electrophoresis cell. In some cases, electrophoresis was carried out in 15 × 15 × 0.075 cm gels in a Hoefer electrophoresis cell. The proteins were stained either with alkaline silver or with Coomassie blue. For immunostaining of the Pgp, the first and second antibodies were 1:250 diluted monoclonal anti-MDR antibody from mouse (C219, Signet, Dedham, MA) and 1:5000 diluted anti-mouse Ig, peroxidase-linked whole antibody from sheep (Amersham), respectively. For yeast plasma membrane H^+-ATPase detection, the first antibody was 1:100 000 diluted polyclonal antiserum from rabbit [30], and the second antibody was 1:5000 diluted anti-rabbit Ig, peroxidase-linked whole antibody from donkey (Amersham). The peroxidase-labeled blots were developed by the enhanced chemiluminescence method, using the Amersham Corp. ECL kit.

2.9. Glycosidase treatment

Samples of 3 μg of the purified Pgp in 0.1% (w/v) DDM and 10 μg of fetuin (Sigma) were mixed and denatured by incubation with 0.5% (w/v) SDS, 1% (v/v) β-mercaptoethanol for 10 min at 50°C. The samples were adjusted to 0.5% (v/v) Nonidet P-40 (Boehringer Mannheim), and 0.1 unit of *N*-glycosidase F (Sigma) or water (to control tube) was added. The samples were incubated at 37°C over night and then analyzed by SDS–PAGE in 7.5% acrylamide gels that were either stained with Coomassie blue or subjected to immunoblotting for the Pgp.

2.10. Estimation of protein

Protein concentrations were determined by the Lowry procedure, as modified by Bensadoun and Weinstein [31] using bovine serum albumin as standard.

2.11. Other materials

The sources of most of the materials have been described previously [17,21,22]. Yeast strain BJ1991 was from the Yeast Genetics Stock Center, University of California, Berkeley, CA. Human Pgp baculovirus infected Sf9 cell membranes were prepared by Mr. Michael Hoben in this laboratory, as described previously [17]. Ni^{2+}-NTA Superflow resin was from Qiagen.

3. Results

Systems for the expression of the *N. crassa* plasma membrane H^+-ATPase and the CFTR in yeast have been previously established in this laboratory [21,22]. On the basis of these experiments, two yeast expression vectors containing the wild-type human *mdr1* cDNA were constructed, as described in Section 2. In these vectors Pgp expression is under the control of the constitutive promoter of the yeast plasma membrane H^+-ATPase. Fig. 1 shows the map of plasmid pQMMDR4, which was used throughout this study. In order to facilitate Pgp purification by Ni^{2+} affinity chromatography, which has proved very useful for the purification of membrane proteins [20,28,32–34], a histidine tag coding for 10 tandem histidines was introduced at the C-terminal end of the Pgp cDNA in pQMMDR4. Plasmid pQMMDR3 is similar without a histidine tag. In addition, all of the three in-frame ATTs upstream from the ATG start codon in the expression vectors were deleted in order to prevent N-terminal peptide extension caused by translation initiation at an upstream in-frame ATT [22]. When the expression vectors were transformed into yeast strain EGY48, which was previously used for expression of the CFTR [22], only a low level of expression was detected, due to substantial proteolytic degradation of the expressed Pgp (data not shown). We therefore switched to the yeast strain BJ1991, which is proteinase-deficient and has been successfully used for the expression of the human AE1 anion exchanger with a high yield [28]. Individual colonies were grown and isolated membranes analyzed for Pgp expression by SDS–PAGE followed by silver staining and immunoblotting. Fig. 2A shows an SDS–PAGE analysis of membranes isolated from

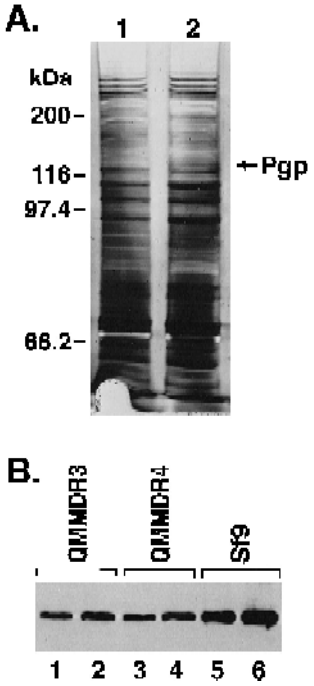

Fig. 2. Expression of the human Pgp in yeast membranes. (A) Silver-stained yeast membrane proteins in a 7.5% acrylamide SDS–PAGE minigel. Lane 1, membranes of control strain RS421-Not (3 μg). Lane 2, membranes of strain QMMDR4 (3 μg). The arrow indicates the 130 kDa Pgp band. (B) Immunoblot analyses of the Pgp expressed in yeast strain BJ1991. Membranes of strains QMMDR3 and QMMDR4 were prepared as described in Section 2 and subjected to SDS–PAGE in a 7.5% acrylamide minigel. Western blotting of the SDS–PAGE gel of the total membranes was then carried out as described in Section 2. The identities of the strains used are indicated above the lanes. Lanes 1 and 3: 20 μg of yeast membranes; lanes 2 and 4: 40 μg of yeast membranes; lanes 5 and 6: 5 and 10 μg of Pgp baculovirus-infected Sf9 insect cell membranes, respectively.

control strain RS421-Not and strain QMMDR4 stained with silver. The band of the Pgp in QMMDR4 membranes (Fig. 2A, lane 2) is easily seen in the position indicated by the arrow. No corresponding band at the position of the Pgp is observed in the control membranes (Fig. 2A, lane 1). Since the Pgp band in such analyses is closely flanked by other proteins, its expression level could not be accurately quantified by direct densitometry. We therefore quantified the Pgp yield in a different way. The amount of the Pgp produced in Sf9 insect cell membranes is larger than that in the yeast membranes and can be estimated by densitometry of silver-stained gels. By this method, the Pgp produced in Sf9 cells represents

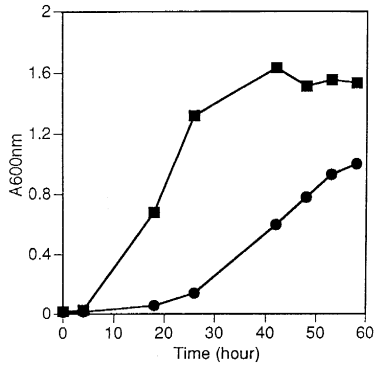

Fig. 3. Influence of expression of the Pgp on yeast cell growth. Time-dependent growth of the strain BJ1991 transformed with the control plasmid pRS421-Not (■) and the Pgp expression plasmid pQMMDR4 (●) were estimated by measuring the optical density (A_{600nm}) in liquid cultures prepared as described in Section 2.

about 3% of the total protein in the light membrane fraction [17]. Fig. 2B shows an immunoblot of membranes isolated from yeast strains QMMDR3 and QMMDR4, and Pgp-expressing Sf9 insect cells. The amount of the Pgp in the membranes of strain QMMDR4 is about 15% of that in Sf9 cell membranes as determined by densitometry of the immunoblots. Thus, the Pgp is produced in strain QMMDR4 in amounts approximately 0.4% of the total yeast membrane proteins, a level similar to that of the CFTR produced in this yeast expression system [22]. Fig. 2B also shows that introduction of a histidine tag to the C-terminus of the Pgp does not interfere with its expression level, because there is no noticeable difference in Pgp expression between strains QMMDR3 and QMMDR4. Since strain QMMDR4 produces a wild-type human Pgp with a histidine tag at its C-terminus for protein purification, all subsequent experiments were carried out primarily with the Pgp produced in strain QMMDR4.

Fig. 3 shows the growth rate of the yeast cells transformed with a control plasmid or plasmid pQMMDR4. The growth rate of cells expressing the Pgp was much slower (doubling time longer than 7 h) than cells transformed with control vector pRS421-Not (doubling time around 3 h). These results indicate that expression of the Pgp can interfere with yeast growth. Similar results were also obtained for the human AE1 anion exchanger [28] and the CFTR (P. Huang and Q. Mao, unpubl. observation), suggesting that this is probably a general effect of forced expression of foreign proteins.

To determine the subcellular distribution of the human Pgp expressed in this yeast system, crude membranes from strain QMMDR4 were fractionated by discontinuous sucrose gradient centrifugation and membrane-containing fractions were analyzed by immunoblotting for the Pgp and the yeast plasma membrane H^+-ATPase. The results of this experiment are shown in Fig. 4. The expressed Pgp and the yeast plasma membrane H^+-ATPase comigrated, and were enriched near the bottom of the gradient, indicating that the majority of the Pgp expressed in this yeast system is directed to the plasma membrane.

The next question of obvious interest regards the functionality of the Pgp expressed in yeast. To address this question we first determined the phenotypic consequence of Pgp expression. Valinomycin, a peptide antibiotic that markedly stimulates the Pgp–ATPase activity [35] and is thus a presumed Pgp substrate, has been shown to be toxic to yeast cells [14,36]. We therefore carried out growth tests to determine whether or not the yeast cells expressing the Pgp display a drug-resistant phenotype. Fig. 5A shows the results of one such experiment. In the absence of valinomycin the control strain RS421-Not grows somewhat better than the Pgp-expressing strain QMMDR4, consistent with the results of Fig. 3 (Fig.

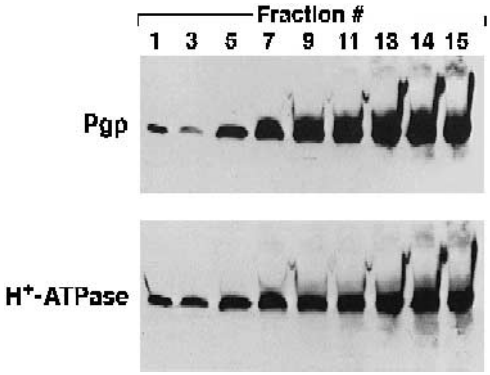

Fig. 4. Subcellular distribution of the expressed Pgp. Shown are immunoblots of the Pgp (upper panel) and the yeast plasma membrane H^+-ATPase (lower panel) on the same blot. Fraction numbers from the top to the bottom of the gradient are shown above each lane. Only the fractions indicated were analyzed. SDS–PAGE was carried out in a 7.5% acrylamide minigel. See Section 2 for details.

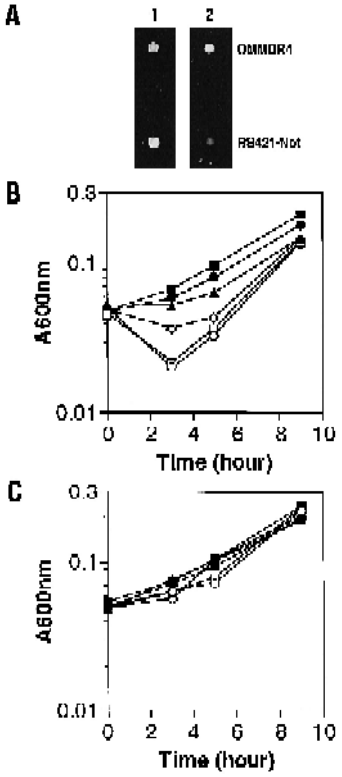

Fig. 5. Expression of the Pgp confers valinomycin-resistance to yeast cells. (A) BJ1991 cells carrying plasmids pQMMDR4 and pRS421-Not were spotted on agar plates without (lane 1) or with (lane 2) 150 μM valinomycin, as described in Section 2. Cell growth was recorded after 48 h at 30°C. The identities of strains QMMDR4 and RS421-Not are indicated. (B) and (C) Effect of valinomycin on the growth of the control strain RS421-Not (B) and Pgp-expressing strain QMMDR4 (C). Cells were grown in synthetic media in the presence of different concentrations of valinomycin at 30°C as described in Section 2. Valinomycin concentrations are 0 (■), 20 μg/ml (●), 55 μg/ml (▲), 110 μg/ml (◇), 130 μg/ml (□) and 165 μg/ml (○). Shown are means of two independent experiments.

5A, lane 1). However, strain RS421-Not grows significantly slower than strain QMMDR4 in the presence of valinomycin (Fig. 5A, lane 2). To obtain a more quantitative estimate of the valinomycin resistance conferred by the expressed Pgp, growth experiments in liquid medium were carried out. Fig. 5B,C summarize the results of these experiments. Significant inhibition of growth of the control strain is seen at 55 μg/ml of valinomycin, and increasing inhibition occurs as the valinomycin concentration is increased. In contrast, growth of the Pgp-expressing strain is unaffected between 0 and 165 μg/ml of valinomycin. Interestingly, the valinomycin effects on the growth of the control strain are eventually overcome in the liquid medium experiments, presumably due to the development of some form of yeast drug resistance [37]. Thus, yeast cells expressing the Pgp acquire a dramatically increased resistance to valinomycin, suggesting that at least some of the Pgp expressed in yeast is functional.

To assess the functionality of the expressed Pgp further it was necessary to solubilize and purify it, because its activity in the crude yeast membrane fraction is masked by a variety of other endogenous ATPase activities. This was greatly aided by the presence of the deca-histidine tag at the C-terminus of the expressed Pgp. We first determined suitable conditions for solubilizing the Pgp from the yeast membranes. Fig. 6 summarizes the most important observations from these experiments. In the absence of NaCl, only 10–20% of the Pgp is solubilized from the yeast membranes by 1% DDM or 1% LPC, respectively, as judged from densitometric analyses of these immunoblots. However, when 400 mM NaCl is added to the solubilization buffer, 70–80% of the Pgp is solubilized by DDM and 80–90% by LPC. Although not shown, octyl-β-glucoside solubilized about 40–50% of the Pgp, but the addition of NaCl had little effect. On the basis of these experiments, initial solubilization of the Pgp is carried out with 1% (w/v) LPC in the presence of 400 mM NaCl.

In initial purification trials the LPC-solubilized Pgp was applied onto a nickel spin column but most

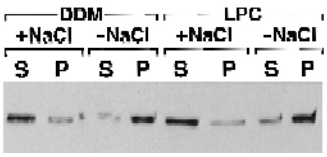

Fig. 6. Solubilization of the Pgp from yeast membranes. QMMDR4 membranes (1 mg of protein) were resuspended in 0.5 ml of solubilization buffer (25 mM Tris, 1 mM PMSF, 2 μg/ml chymostatin, leupeptin, pepstatin A and aprotinin, 1 mM β-mercaptoethanol, and 20% (w/v) glycerol, pH 8.0 with HCl) containing 1% (w/v) LPC or 1% (w/v) DDM with or without 400 mM NaCl as indicated. Samples were incubated at 4°C with gentle stirring for 20 min and centrifuged for 45 min at 100 000 $\times$ g (4°C). The supernatants were collected, and the pellets from each sample were thoroughly resuspended in 0.5 ml of solubilization buffer lacking detergent. The same volume (20 μl) of supernatants (S) and pellets (P) was disaggregated and subjected to SDS–PAGE in a 7.5% acrylamide minigel, and processed for immunoblotting of the Pgp. See Section 2 for additional details.

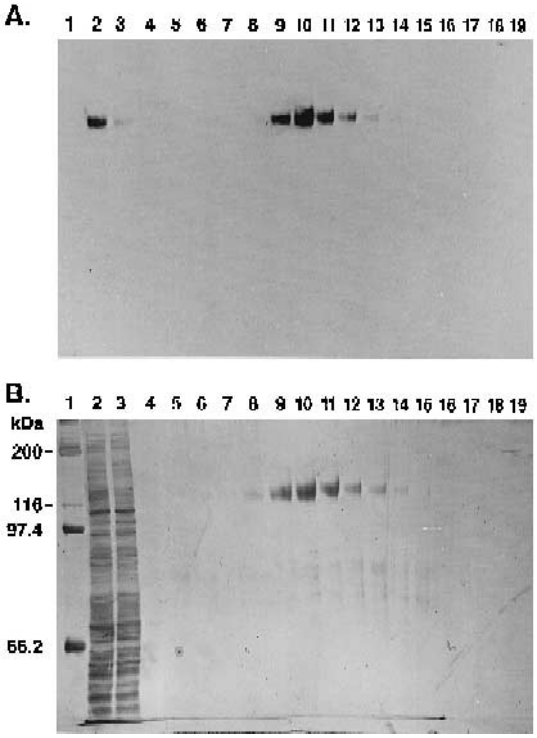

Fig. 7. Purification of the yeast-expressed Pgp by Ni^{2+} affinity chromatography. QMMDR4 membranes (20 mg of protein) were solubilized with LPC, the supernatant collected, and the solubilized Pgp was purified by Ni^{2+} affinity chromatography as described in Section 2. Lane 1, molecular weight markers; lane 2, starting material (LPC-solubilized extract); lane 3, flow-through; lane 4, pool of wash with 30 mM imidazole buffer; lane 5, pool of wash with 60 mM imidazole buffer; lanes 6–19, fractions eluted with 250 mM imidazole buffer. Aliquots of the samples (25 μl for each lane) were subjected to SDS–PAGE in 7.5% acrylamide, $15\times15\times0.075$ cm gels that were then either transferred to nitrocellulose membrane for immunoblotting of the Pgp (A) or stained with alkaline silver (B). The molecular masses of the protein standards are indicated to the left of the gel in panel B.

of the Pgp failed to bind to the column. However, it has been observed that incubation of solubilized membrane proteins with the Ni^{2+}-NTA resin for about 1 h at 4°C can significantly improve protein binding [33] and, when the LPC-solubilized Pgp was applied to the Ni^{2+}-NTA resin in this way, binding was greatly improved. Fig. 7 shows the results of a typical Pgp purification experiment. More than 90% of the solubilized Pgp is bound to the nickel resin, as judged by densitometry of the immunoblots of the starting material (Fig. 7A, lane 2) and the flow-through after protein binding (Fig. 7A, lane 3). After

Table 1
Protein recovery during the LPC solubilization and the Ni^{2+} affinity chromatography of the Pgp

Steps	Protein (mg)	Yield of Pgp (%)
Crude membrane	20	100
LPC-solubilized extract	13	81
Flow-through	11	–
Purified Pgp	0.05	63

Crude membranes (20 mg of protein) were prepared as described in Section 2. The yield of Pgp at each step is calculated relative to the overall Pgp present in the plasma membranes (100%).

elution with 250 mM imidazole, the Pgp is concentrated in just a few fractions (Fig. 7A, lanes 9–12). Panel B of Fig. 7 shows a silver stained SDS–PAGE gel analyses of the same fractions. The Pgp emerges from the Ni^{2+}-NTA column in nearly pure form. Table 1 indicates the yields of protein present in the various fractions of the experiment of Fig. 7. About 50 μg of purified Pgp protein is obtained from 20 mg of crude yeast membranes. From quantitative immunoblots, we estimate that 20 mg of such membranes contain about 80 μg of the Pgp. Thus, the recovery of the Pgp solubilization and purification procedures is greater than 60%. In addition to its remarkable ease and effectiveness, the Ni^{2+}-NTA purification procedure provides an additional advantage in that it readily facilitates detergent exchange. Thus, since LPC tends to precipitate on standing at

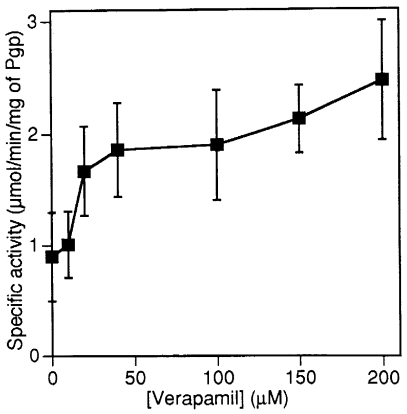

Fig. 8. Verapamil-stimulated ATPase activity of the purified Pgp. ATPase activity of the purified Pgp was determined in the presence of *E. coli* lipids and several concentrations of verapamil, as described in Section 2. Shown are means and standard deviations of five independent experiments.

4°C, we routinely exchanged it for DDM during the Ni^{2+} affinity chromatography step.

The availability of highly purified Pgp allows a direct assessment of its functionality. A reliable index of the functionality of the Pgp is its verapamil-stimulated ATPase activity [17]. Fig. 8 shows the verapamil concentration dependence of the ATPase activity of the purified wild-type Pgp. In the absence of verapamil, the purified Pgp has an ATPase activity of about 0.9 μmol/min per mg of Pgp. In the light of recent data supporting the notion that the Pgp is a lipid translocase of broad specificity [38], the activity we detect in the absence of verapamil is most likely due to the presence of other Pgp substrates, such as the *E. coli* lipids added to the assay or the detergents used in the purification procedure. With increasing concentrations of verapamil, the ATPase activity increases 2.8-fold to 2.5 μmol/min per mg of Pgp, which is comparable to the highest activities reported from other laboratories [11,12,20,39,40].

The immunoblot analyses shown in Fig. 2B shows that there is no noticeable mobility difference between the Pgp expressed in yeast and that produced in Sf9 insect cells, suggesting that the Pgp produced in yeast is not substantially glycosylated. To assess further whether or not the Pgp expressed in yeast is glycosylated, the effect of *N*-glycosidase F treatment on the purified Pgp was determined. In this experiment, the Pgp purified as described above and a glycosylated control protein, fetuin, were mixed and together treated with *N*-glycosidase F as previously described [28], and the electrophoretic mobility of the Pgp with and without *N*-glycosidase F treatment was assessed by SDS–PAGE. Fig. 9A shows such a gel stained with Coomassie blue and Fig. 9B shows the results of an immunoblot. Whereas fetuin exhibited a marked mobility increase upon the *N*-glycosidase F treatment, the mobility of the Pgp was unaltered, suggesting that the Pgp expressed in yeast is not glycosylated. This conclusion is in agreement with the observations of Kuchler and Thorner [14]. Finally, the purified Pgp has an electrophoretic mobility corresponding to a molecular mass of about 130 kDa, which is in good agreement with the molecular mass of the unglycosylated human Pgp predicted from the gene sequence.

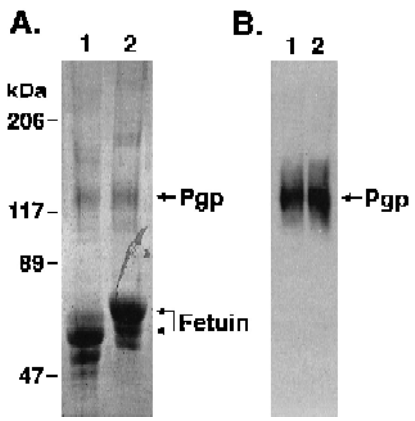

Fig. 9. Effect of *N*-glycosidase F treatment on the purified Pgp produced in yeast. A mixture of the purified Pgp and glycosylated control protein, fetuin, were treated with or without *N*-glycosidase F and analyzed by SDS–PAGE, as described in Section 2. (A) Coomassie blue-stained SDS–PAGE gel. (B) Immunostaining for the Pgp. Electrophoresis was carried out in a 7.5% acrylamide, $15 \times 15 \times 0.075$ cm gel. Positions of the Pgp and fetuin before treatment are indicated by arrows. Lane 1, sample treated with *N*-glycosidase F; lane 2, control without glycosidase treatment.

4. Discussion

The results we have obtained in this study demonstrate that the human Pgp can be functionally expressed in *S. cerevisiae* in significant yields. It is estimated that the recombinant Pgp is produced in amounts approximately 0.4% of the total yeast membrane protein. Several factors contributed to this substantial level of Pgp expression. First, the expression system used allows for a high gene copy number, due to a multicopy plasmid and a high transcriptional activity owing to the strong constitutive yeast plasma membrane H^+-ATPase promoter [23]. Although not studied here, another factor possibly contributing to this yield of the Pgp is the optimized arrangement of the bases in the promoter region just preceding the ATG start codon of the Pgp. As we have previously shown, deletion of in-frame ATTs upstream from the ATG start codon dramatically increases the level of expression of the CFTR in this system [22]. For this reason, plasmids pQMMDR3 and pQMMDR4 were constructed without these upstream ATTs to mimic the CFTR expression plasmid, pHCFTRW, described by Huang et al. [22]. The choice of the host yeast strain used for these studies was also an important

factor for obtaining significant levels of expression of the Pgp. Initially, the Pgp expression plasmid was transformed into the yeast strain EGY48, which was previously used for the expression of the CFTR [22]. However, the expression level of the Pgp in strain EGY48 was low and variable, due to proteolytic degradation of the expressed Pgp by endogenous yeast proteinases. The use of the yeast strain BJ1991, which is proteinase-deficient [41], led to a substantial decrease in Pgp degradation and a concomitant increase in intact Pgp production.

Pgp has been studied exhaustively for many years in living organisms and at the cellular and subcellular levels. More recently, it has become possible to study certain enzymatic features of this important molecule at the biochemical level [17,10,9–12,15,20,42,43]. However, at present, little is known regarding the structure of Pgp and the molecular mechanism by which it confers multidrug resistance to cells in which it is over-expressed. Progress in this regard will require the development of procedures for isolating Pgp in large quantities and the determination of its structure at low resolution by biochemical means and, ultimately, at higher resolution by electron crystallography of 2-dimensional crystals [44] or X-ray crystallography of 3-dimensional crystals [45–47]. A variety of systems are available for Pgp production, including the drug-resistant cultured cell systems [9–12] and heterologous expression systems in Sf9 insect cell [16,17], *E. coli* [18,19], yeast [13–15,48], and transiently transfected HEK293 cells [20]. However, only a few of these are capable of producing the high levels of the Pgp needed for extensive biochemical and biophysical studies. Of these, the system using drug-resistant cultured cells may be highly useful, but a major concern with these systems is the extensive level of Pgp glycosylation, which may preclude crystallization. The expensive and tedious procedures for maintaining the cultured cells are another significant drawback with these systems. Another Pgp production system currently available is the Sf9 cultured insect cell system, which offers yields of the Pgp up to about 3% of the insect cell light membrane fraction [17]. In addition to this high yield, this system offers an under-glycosylated Pgp, which may be easier to crystallize. However, a major drawback of this system is the expense and labor of the insect cell growth and maintenance, which makes obtaining large amounts of starting material for Pgp purification difficult, at best.

Heterologous Pgp expression systems using microorganisms can potentially obviate the above mentioned problems with cultured animal and insect cell expression systems. Several such systems have been reported [13–15,18,19,48] but the expression levels are difficult to estimate from the data provided. The Pgp expression system described here produces Pgp at levels somewhat less than that of the cultured animal and insect cell systems, on a milligram of membrane protein basis. However, the low cost and ease with which massive quantities of yeast cells can be grown more than compensate for the lower yield compared to the other systems. The Pgp produced in yeast is also apparently unglycosylated, and site-directed mutants can readily be generated. Moreover, with the deca-histidine tag attached, the recombinant Pgp is extremely easy to purify. Finally, the Ni^{2+} chelate chromatography step offers a easy means for detergent exchange, which may be indispensable for Pgp crystallization experiments.

In summary, we have expressed the human Pgp with substantial yields in a yeast system used previously to produce recombinant transport ATPases and the CFTR. We have also purified the Pgp from the yeast membranes to near homogeneity in functional form using Ni^{2+} affinity chromatography. This yeast expression and purification system should provide an excellent source of material for biochemical and biophysical studies aimed at elucidating the molecular structure and mechanism of drug transport catalyzed by the Pgp. The results obtained may be also of value for the heterologous expression and purification of other integral membrane proteins, including other medically important mammalian ABC transporters.

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References

[1] J.A. Endicott, V. Ling, Annu. Rev. Biochem. 58 (1989) 137–181.
[2] I.B. Roninson (Ed.), Molecular and Cellular Biology of Multidrug Resistance in Tumor Cells, Plenum Press, New York, 1991.
[3] M.M. Gottesman, I. Pastan, Annu. Rev. Biochem. 62 (1993) 385–427.
[4] P. Gros, E. Buschman, Int. Rev. Cytol. 137 (1993) 169–197.
[5] P.D. Roepe, Biochim. Biophys. Acta 1241 (1995) 385–406.
[6] C.F. Higgins, I.D. Hiles, G.P.C. Salmond, D.R. Gill, J.A. Downie, I.J. Evans, I.B. Holland, L. Gray, S.D. Buckel, A.W. Bell, M.A. Hermodson, Nature 323 (1986) 448–450.
[7] S.C. Hyde, P. Emsley, M.P. Hartshorn, D.R. Gill, R.E. Hubbard, C.F. Higgins, Nature 346 (1990) 362–365.
[8] C.S. Mimura, S.R. Holbrook, G.F.-L. Ames, Proc. Natl. Acad. Sci. USA 88 (1991) 84–88.
[9] C.A. Doige, X. Yu, F.J. Sharom, Biochim. Biophys. Acta 1109 (1992) 149–160.
[10] S.V. Ambudkar, I.H. Lelong, J. Zhang, C.O. Cardarelli, M.M. Gottesman, I. Pastan, Proc. Natl. Acad. Sci. USA 89 (1992) 8472–8476.
[11] A.B. Shapiro, V. Ling, J. Biol. Chem. 269 (1994) 3745–3754.
[12] I.L. Urbatsch, M.K. Al-Shawi, A.E. Senior, Biochemistry 33 (1994) 7069–7076.
[13] M. Raymond, P. Gros, M. Whiteway, D.Y. Thomas, Science 256 (1992) 232–233.
[14] K. Kuchler, J. Thorner, Proc. Natl. Acad. Sci. USA 89 (1992) 2302–2306.
[15] S. Ruetz, M. Raymond, P. Gros, Proc. Natl. Acad. Sci. USA 90 (1993) 11588–11592.
[16] U.A. Germann, M.C. Willingham, I. Pastan, M.M. Gottesman, Biochemistry 29 (1990) 2295–2303.
[17] B. Sarkadi, E.M. Price, R.C. Boucher, U.A. Germann, G.A. Scarborough, J. Biol. Chem. 267 (1992) 4854–4858.
[18] E. Bibi, P. Gros, H.R. Kaback, Proc. Natl. Acad. Sci. USA 90 (1993) 9209–9213.
[19] O. Beja, E. Bibi, Proc. Natl. Acad. Sci. USA 93 (1996) 5969–5974.
[20] T.W. Loo, D.M. Clarke, J. Biol. Chem. 270 (1995) 21449–21452.
[21] S.K. Mahanty, U.S. Rao, R.A. Nicholas, G.A. Scarborough, J. Biol. Chem. 269 (1994) 17705–17712.
[22] P. Huang, K. Stroffekova, J. Cuppoletti, S.K. Mahanty, G.A. Scarborough, Biochim. Biophys. Acta 1281 (1996) 80–90.
[23] J.M. Villalba, M.G. Palmgren, G.E. Berberian, C. Ferguson, R. Serrano, J. Biol. Chem. 267 (1992) 12341–12349.
[24] J. Sambrook, E.F. Fritsch, T. Maniatis, Molecular Cloning, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989.
[25] S.K. Mahanty, G.A. Scarborough, J. Biol. Chem. 271 (1996) 367–371.
[26] N. Kioka, J. Tsubota, Y. Kakehi, T. Komano, M.M. Gottesman, I. Pastan, K. Ueda, Biochem. Biophys. Res. Commun. 162 (1989) 224–231.
[27] R.M. Horton, Z. Cai, S.N. Ho, L.R. Pease, BioTechniques 8 (1990) 528–535.
[28] I. Sekler, R. Kopito, J.R. Casey, J. Biol. Chem. 270 (1995) 21028–21034.
[29] W. Wray, T. Boulikas, P. Wray, R. Hancock, Anal. Biochem. 118 (1981) 197–203.
[30] E. Capieaux, C. Rapin, D. Thines, Y. Dupont, A. Goffeau, J. Biol. Chem. 268 (1993) 21895–21900.
[31] A. Bensadoun, D. Weinstein, Anal. Biochem. 70 (1976) 241–250.
[32] U. Waeber, A. Buhr, T. Schunk, B. Erni, FEBS Lett. 324 (1993) 109–112.
[33] Q. Mao, T. Schunk, B. Gerber, B. Erni, J. Biol. Chem. 270 (1995) 18295–18300.
[34] B. Loddenkoetter, B. Kammerer, K. Fisher, U.-I. Fluegge, Proc. Natl. Acad. Sci. USA 90 (1993) 2155–2159.
[35] U.S. Rao, G.A. Scarborough, Mol. Pharmacol. 45 (1994) 773–776.
[36] S. Ruetz, M. Brault, C. Kast, C. Hemenway, J. Heitman, C. Grant, S.P.C. Cole, R.G. Deeley, P. Gros, J. Biol. Chem. 271 (1996) 4154–4160.
[37] M. Kolaczkowski, M. van der Rest, A. Cybularz-Kolaczkowska, J.-P. Soumillion, W.N. Konings, A. Goffeau, J. Biol. Chem. 271 (1996) 31543–31548.
[38] A. van Helvoort, A.J. Smith, H. Sprong, I. Fritzsche, A.H. Schinkel, P. Borst, G. van Meer, Cell 87 (1996) 507–517.
[39] I.L. Urbatsch, A.E. Senior, Arch. Biochem. Biophys. 316 (1995) 135–140.
[40] R. Liu, F.J. Sharom, Biochemistry 35 (1996) 11865–11973.
[41] E.W. Jones, Methods Enzymol. 194 (1991) 428–453.
[42] T.W. Loo, D.M. Clarke, J. Biol. Chem. 269 (1994) 7750–7755.
[43] I. Urbatsch, B. Sankaran, S. Bhagat, A.E. Senior, J. Biol. Chem. 270 (1995) 26956–26961.
[44] W. Kühlbrandt, D.N. Wang, Y. Fujiyoshi, Nature 367 (1994) 614–621.
[45] J. Deisenhofer, O. Epp, K. Miki, R. Huber, H. Michel, Nature 318 (1985) 618–624.
[46] S. Iwata, C. Ostermeier, B. Ludwig, H. Michel, Nature 376 (1995) 660–669.
[47] T. Tsukihara, H. Aoyama, E. Yamashita, T. Tomizaki, H. Yamaguchi, K. Shinzawa-Itoh, R. Nakashima, R. Yaono, S. Yoshikawa, Science 269 (1995) 1069–1074.
[48] S. Ruetz, P. Gros, J. Biol. Chem. 269 (1994) 12277–12284.