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ORIGINAL ARTICLE

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In vitro translation of a 2.3-kb splicing variant of the hamster pgp1 gene whose presence in transfectants is associated with decreased drug resistance

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Abstract *Purpose*: P-glycoprotein (P-gp), a product of the Chinese hamster pgp1 gene, confers multidrug resistance to mammalian cells in which it is overexpressed either by transfection or as a result of drug selection. It is encoded by a 4.3-kb mRNA and in its unglycosylated form has a predicted molecular weight of approximately 141 kDa. When a cDNA containing this sequence is transfected into drug-sensitive Chinese hamster lung cells and is expressed under the control of the β-actin promoter, both the full-length 4.3-kb mRNA and a 2.3-kb transcript are produced. The latter results from a splicing event that utilizes near consensus 5′ and 3′ splicing signals resident in the full-length mRNA, and it has also been found to be present in cell lines that express the native gene. Therefore, it is a splicing product of pgp1 per se. This report is concerned with the biological relevance of this transcript. *Methods*: In vitro transcription and translation experiments were used to show that the putative open reading frame of the 2.3-kb transcript encodes a novel 57-kDa protein ($p57^{pgp1}$) that contains transmembrane domains 9–12 and the C-terminal ATP binding fold of P-gp. To elucidate the function of $p57^{pgp1}$, expression vectors containing cDNAs representing (1) the 2.3-kb transcript, (2) the full-length 4.3-kb mRNA, and (3) a splice-disabled 4.3-kb transcript in which production of the 2.3-kb transcript is eliminated by an in-frame mutation at the 3′ splice site, were constructed and transfected into DC-3F cells. Additional expression vectors in which $p57^{pgp1}$ represented the N-terminus of a green fluorescent protein fusion construct were also prepared and used for transient expression studies. *Results*: Overexpression of the 2.3-kb transcript alone did not confer multidrug resistance. Transfectants in which both the 4.3-kb transcript and the 2.3-kb transcript were present, compared with transfectants in which no 2.3-kb transcript was expressed, but in which the level of expression of the 4.3-kb mRNA alone was the same, showed little change in cross-resistance pattern. However, the overall level of resistance in the latter cells was increased by approximately twofold. Hence the presence of the 2.3-kb transcript was associated with a decrease in drug resistance. In vitro transcription and translation experiments and transient expression studies indicate that $p57^{pgp1}$ can be expressed both in vitro and in vivo. *Conclusion*: These results demonstrate that a splicing variant of pgpl contains an open reading frame capable of translation in vitro and in vivo and suggest that alterations in splicing may contribute both directly and indirectly to the overall mechanism of pgp1-mediated multidrug resistance in CHL cells.

Key words Multidrug resistance · Pgp1 · Splicing · GFP fusion proteins

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Introduction

P-glycoprotein (P-gp) is a member of the ATP-binding cassette family of membrane transporters [1], whose overexpression has been shown to be closely associated with the acquisition of multidrug resistance (MDR) in a wide variety of selected cell lines and tumors [2, 3]. In rodents there are three isoforms of P-gp, and it is pgp1, the hamster homolog of human MDR1, and mouse

mdr1a (mdr3) that has been extensively studied, and has been demonstrated to mediate drug efflux in MDR cells [4]. While the function of pgp1 as an ATP-driven efflux pump in MDR cell lines and transfectants has been well documented, there remains a question as to its role in nondrug-resistant, i.e. normal, cells. Indeed, while it has been demonstrated using knockout mice that the ability of drugs to enter the brain is heightened by the absence of mdr1a from the blood–brain barrier, and that serum clearing times for certain anticancer drugs are prolonged as well [5, 6], mdr1a (−/−) mice are apparently healthy, fertile and display normal life expectancies [7]. Hence the absence of mdr1a is not lethal. This coupled with reports that have associated MDR1/mdr1a function in nondrug-resistant cells with peptide transport [8], cholesterol biosynthesis and esterification [9, 10], chloride channel activity [11–13], and lipid transport [14], suggest that pgp1 homologs function in roles other than those associated with drug resistance.

One way in which pgp1 might serve multiple functions would be if differential splicing led to the production of different forms of the protein [15]. Different splicing variants of human mdr3 transcripts have been isolated from human liver cDNA libraries [16], and several reports have identified the presence of small pgp1-related transcripts in a variety of selected and transfected cell lines [17–22]. Moreover, there are also reports of small mdr1-related proteins expressed in drug-resistant [22], and nondrug-resistant cells [23], but no direct evidence for the association of a specific splicing event with the presence of a specific protein has yet been provided.

We have previously reported the cloning of two splicing variants of Chinese hamster pgp1 mRNA from cDNA libraries prepared from polysomal poly(A)$^+$ RNA of MDR DC-3F/ADX cells [4]. The cDNA inserts of plasmids pADX124 and pADX185 represent these mRNA splicing variants, and RNAase protection and S1 nuclease assays have confirmed their presence in different MDR cell lines. The pADX124 variant was found to also be present in parental drug-sensitive DC-3F cells as well. The splicing reaction that generates the RNA encoded by the insert in pADX185 eliminates exon four of the pgp1 primary transcript resulting in a loss of the normal reading frame [4]. The splicing reaction responsible for the transcript represented by the pADX124 insert maintains the open reading frame of the normal full-length pgp1 mRNA but eliminates the last 18 nucleotides of exon 4, and all of exons 5 through 18. This results in the joining of the normal N-terminus of the protein with its C-terminal four transmembrane α helices and ATP-binding domain [4]. A diagram of the protein encoded by the pADX124 cDNA insert as predicted from its nucleotide sequence is shown in Fig. 1. The protein contains 538 amino acids with a combined molecular weight of approximately 59 kDa.

We report here our efforts to demonstrate that the pADX124 insert represents a functional mRNA as defined by its ability to direct the synthesis of a specific protein in vitro, and to allow the expression of a green fluorescence protein (GFP) fusion construct in vivo. We also provide evidence for its role in mediating MDR in transfected cell lines.

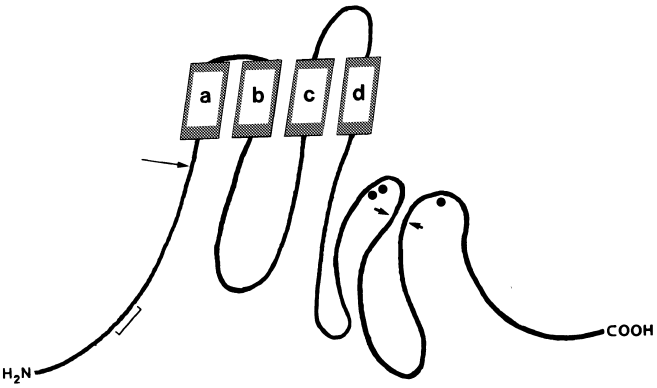

Fig. 1 Diagram of the protein predicted to be encoded by ADX124. For ease of reference and familiarity, the protein is depicted as having maintained some of the core structural features that are thought to be associated with pgp1, i.e. N- and C-termini that are cytosolic as well as an ATP-binding fold (*short arrows*). The four predicted transmembrane domains that correspond to transmembrane domains 9–12 of pgp1 are indicated by the letters *a* to *d*. The *long arrow* indicates the location of the only potential N–glycosylation site (Asn-X-Ser/Thr) in the peptide, and the *bracket* shows the location of the splice junction. The locations of the C219 (•) and C494 (••) epitopes are also indicated

Materials and methods

Site-specific mutagenesis and construction of expression vectors

Because the original cDNA clone, pUC8-ADX124 (pADX124), had been isolated from a GC-tailed cDNA library [4], it was necessary to alter the ends of the insert to permit facile construction of a eukaryotic expression vector. To construct the 5′ end of the insert, polymerase chain reaction (PCR), along with the presence of a unique Af1III site located within the body of the pADX124 cDNA, were used to generate a 687-bp fragment whose 5′ end was modified to contain a BamHI site and whose 3′ end was the original Af1III site. To obtain the 3′ end of the pADX124 insert advantage was taken of the fact that the original inserts of pADX124 and pADX165 [4] share the same 3′ ends and that the pADX165 insert had been previously engineered to contain BamHI ends. Therefore, pGA-1 [24] was digested with Af1III and BamH1 and the resulting 1.4-kb BamHI-Af1III fragment representing the 3′ end of the insert was purified by gel electrophoresis. The 687-bp and 1.4-kb BamH1-Af1III fragments were then cloned into the BamHI site of pGEM4Z (Promega, Madison, Wis.) using a three-way ligation strategy. The final construct was verified by restriction mapping and DNA sequencing. The insert from this clone was then removed by digestion with BamHI and recloned into the BamHI site of the mammalian expression vector pHβneo, as described previously [25]. The orientation of the insert in the final construct, pHβneo124, was verified by DNA sequencing.

To prevent full-length pgp1 mRNA from serving as a splicing substrate, the AG dinucleotide located at nucleotide position 2406 that identifies the position of the putative 3′ splice site in this transcript [4], and that is known to mark the 3′ ends of U2-type introns [26], was converted to AA. This alteration does not change the resulting amino acid sequence since cAG and cAA both code for glutamine. To replace G with A at position 2407, we used an Alter-A-Site mutagenesis kit (Promega) and the oligonuclotide 5′-CTTTTATTACATTTTTTCT-TCAAGGCTTCACAT-3′ in

which the underlined A indicates the site of mutagenesis. The full-length pgp1 cDNA insert from pGA-1 [24] was removed by BamHI digestion and cloned into the vector provided with the kit. Further manipulations were carried out as prescribed by the vendor. Clones containing the correct mutation were identified by restriction mapping and DNA sequence analysis and the insert from one of them removed from the vector and cloned into the BamHI site of pHβneo, as described previously, [25]. The orientation of the insert within the resulting expression vector, phβneoCAA, was confirmed by nucleotide sequence analysis.

Development of stable transfectants, ED_{50} determinations and Northern blot analysis

Expression vectors were transfected into parental drug-sensitive DC-3F cells via calcium phosphate precipitation as described previously [25]. G418 selection was then applied and the surviving colonies isolated and expanded. At no time during the selection process were colonies exposed to MDR drugs. G418-positive clones were tested for the expression of P-gp mRNA and protein via Northern and Western blot analysis, respectively. Those colonies found to be expressing P-gp were then analyzed for MDR by determining their respective ED_{50} values for each of the four drugs actinomycin D, colchicine, vincristine and daunorubicin, as described elsewhere [27]. Total RNA was extracted using the RNAzol method, and Northern blots were performed using a full-length [^{32}P]-labeled pgp1 cDNA probe, as described previously [25]. mRNA expression levels were determined using an Alpha Innotech Image Analysis System (San Leandro, CA).

Western blot analysis

Cell lysates were prepared from cell lines and transfectants as described previously [27]. Western blots were carried out using 40 μg of total protein separated on SDS-polyacrylamide gels. After transfer of the protein to nitrocellulose membranes, the blots were analyzed using the monoclonal antibodies C219 and C494 [28], purchased from Signet (Dedham, MA), and quantified by image analysis.

In vitro transcription and translation

RNA transcripts were produced by in vitro transcription using the Sp6 RiboMAX large-scale transcription system from Promega. The full-length cDNAs for pgp1 and ADX124 were cloned into the BamHI site of pGEM4Z in sense orientation to the Sp6 promoter. An additional clone was prepared as a negative control that contained full-length pgp1 in an antisense orientation. Conditions for the in vitro transcription reactions were those provided by the vendor. The resulting capped RNA transcripts (3 μg of each) were used as templates for in vitro translation using the Flexi Rabbit Reticulocyte Lysate System, also from Promega. Luciferase mRNA supplied with the kit was used as a positive control for translation per se, while both positive and negative glycosylation controls were provided with the microsomal membrane preparations used in these studies (Promega). In vitro translation was carried out in the presence or absence of microsomal membranes, RNasin, and either with nonradioactive methionine or ^{35}S-labeled methionine as described by the vendor. Translation products were separated by electrophoresis through 7.5% polyacrylamide gels. After Coomassie blue staining, radioactive gels were dried and then exposed to Kodak X-Omat film. Nonradioactive gels were prepared for Western blot analysis as described previously [27].

Transient expression studies with GFP fusion constructs

The insert from pHβneo124 was removed by digestion with BamHI and recloned into pGEM4Z. The plasmid was then linearized with Eco47III, which cuts within the insert two bases 5′ of the TAG stop codon and leaves blunt ends. BamHI linkers were added and the linearized plasmid then cut into two pieces with BamHI. The resulting 2-kb DNA fragment from which the original stop codon had now been removed, was isolated by gel electrophoresis and cloned into the BamHI site of pEGFP-N2, an N-terminal GFP fusion vector from Clonetech (Palo Alto, CA), in frame with the vector's GFP coding region. The construct was verified by DNA sequencing. In this configuration expression of GFP relies on the start codon and open reading frame provided by the insert. A second vector containing the 2-kb insert cloned in the opposite orientation was also constructed and served as a negative control. Expression of pEGFP-N2 itself was used as the positive control.

The GFP vectors were transfected into DC-3F cells using Lipofectin reagent as described by the vendor (GIBCO BRL, Life Technologies, Rockville, MD). Cells were grown in chamber slides (Nalge, Nunc Int., Rochester, NY), and after 72 h, cells were fixed by incubation for 30 min at room temperature in 2 ml of PBS containing 4% paraformaldehyde as suggested by the vendor (Clontech). Transient expression of GFP was monitored using a Zeiss fluorescent microscope with filter set #487909 with a 450–490 nm bandpass excitation filter, a 510 nm dichroic reflector and a 520–750 nm longpass emission filter, also as suggested by the vendor. A minimium of 200 individual cells were analyzed per assay.

Results

Expression of spliced variants of pgp1

We have previously shown by sequence analysis that the full-length 4.3-kb mRNA transcript encoded by the hamster pgp1 gene, and cloned in pADX165, contains a near consensus eukaryotic 5′ splice signal within exon four at base 204, and a very well conserved 3′ splice signal at nucleotide position 2407, at the 3′ end of exon 18 [4]. More importantly, the RNA transcript represented by the cDNA insert in pADX124 was generated by a splicing event that utilized these two processing sites, suggesting that it originated as an alternate splicing product of the pgp1 gene primary transcript [4]. Demonstration that transfection of the full-length pgp1 cDNA into DC-3F cells leads to the overexpression of both the full-length mRNA and near equal amounts of a 2.3-kb transcript [25], however, strongly suggests that the full-length transcript could serve as a splicing substrate, and that in transfected cells, it was the origin of the 2.3-kb RNA molecule.

To determine whether the presence of the 2.3-kb transcript affected MDR in cells expressing a pgp1 transgene, DC-3F cells were transfected with pHβneo124, pHβneoCAA and pLK212s-17 [25], which acted as a positive control for the splicing event. An additional set of transfections was also carried out in which cells were cotransfected with equal amounts of pHβneo124 and pHβneoCAA. Neomycin-resistant colonies were isolated and expanded, and total RNA was prepared for Northern blot analysis. As seen in Fig. 2, cells expressing the insert from pLK212s-17 (lane 1) displayed similar amounts of both the 4.3- and 2.3-kb pgp1 transcripts, while the pHβneoCAA transfectants (Fig. 2, lane 3) expressed only the 4.3-kb transcript, but did so to a level equal to that observed in pLK212S-17. Hence, conversion of the AG 3′ splicing signal in the ADX165 primary transcript to AA effectively eliminates splicing. The 4.3-kb transcript was also expressed in

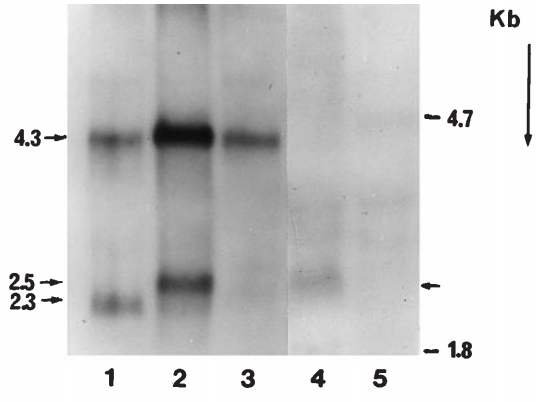

Fig. 2 Northern blot analysis of transfectants. Total RNA (10 μg) from each cell line was electrophoresed in a 1.2% agarose/formaldehyde gel, transferred to nitrocellulose and hybridized with a full-length pgp1 cDNA probe that had been oligo-labeled with [^{32}P] as described previously [4]. The resulting autoradiograms were then quantified by image analysis using software provided by the vendor (*lane 1* pLK212S-17, a transfectant expressing the wild-type pgp1 ADX165 cDNA [23], *lane 2* pHβneo124-pHβneoCAA cotransfectant, *lane 3* pHβneoCAA, *lane 4* pHβneo124, *lane 5* DC-3F)

cotransfectants (Fig. 2, lane 2). However, in the pHβneo124 transfectants (Fig. 2, lane 4), and in the cotransfectants as well (lane 2), a 2.5-kb rather than a 2.3-kb transcript was observed.

All of the inserts used here contained the normal polyadenylation signal found in pgp1 mRNA, i.e. AAUUAA [4], and, as part of the vector, the SV40 polyadenylation signal, AAUAAA, which is located 162 nucleotides downstream. Hence, the approximately 200 nucleotide difference in length between the 2.3- and 2.5-kb transcripts most likely reflects differential use of these two polyadenylation signals. When splicing occurs (Fig. 2, lane 1) polyadenylation of the small transcript takes place at the endogenous site, generating the 2.3-kb molecule, whereas in the absence of splicing [lanes 2 and 4] polyadenylation of the small transcript takes place at the SV40 site, thus generating the 2.5-kb molecule. A similar situation may also exist during processing of the large transcript, since the 4.3-kb molecule in Fig. 2 lane 1 does appear to be somewhat smaller than it is in lanes 2 and 3.

Taken together, these results demonstrate that splicing of the 4.3-kb pgp1 transcript is responsible for the production of the 2.3-kb transcript in transfectants, and that mutation of the endogenous 3′ splice signal found in the full-length transcript prevents expression of the smaller transcript. They also suggest that differential utilization of the polyadenylation signals present in these constructs is associated with splicing of the primary transcript encoded by the insert of pADX165.

Expression of the 2.3-kb transcript does not alter the pattern of cross-resistance but is associated with a decrease in the level of drug resistance

Clones expressing the various constructs were expanded and their levels of resistance to four MDR drugs determined. ED_{50} values (the amount of drug necessary to cause a 50% reduction of cell growth in 72 h) were determined for each of four drugs, and the fold resistance calculated by comparison with drug-sensitive DC-3F cells. The results are shown in Table 1 and Fig. 3. With the exception of colonies expressing pHβneo124, alone all of the transfectants displayed resistance to each of the drugs tested, and all exhibited similar cross-resistance profiles. The inability of the pHβneoCAA transcript to be spliced, therefore, did not affect its ability to confer MDR. However, the level of resistance displayed by the pLK212S-17 transfectants was less than half that noted in cells expressing the splicing mutant in which the 2.3-kb transcript was not present, even though both expressed similar amounts of the full-length 4.3-kb mRNA (Fig. 2). Western blot analysis of crude membrane preparations from each transfected cell line and the cotransfectant as well confirmed that the 4.3-kb transcript level accurately reflected the amount of P-gp actually present (data not shown).

These results demonstrate that while expression of the 2.3-kb transcript is not sufficient to confer MDR and its presence is not required for P-gp to confer MDR, its expression appears to be associated with an overall decrease in the level of drug resistance. This is also consistent with results seen in the cotransfectants in which both the 2.3-kb transcript and the 4.3-kb mRNA are expressed. In this case the level of expression of the 4.3-kb mRNA was twice that seen in the pHβneoCAA transfectants, but the level of resistance was only marginally increased.

Table 1 Drug-resistance properties of transfectants. ED_{50} values were determined for DC-3F cells [23] and transfectants for each of the durgs indicated. The numbers shown represent the average and standard deviation from three experiments carried out in parallel. The drug-resistance levels of the tranfectants were determined by comparison with DC-3F whose ED_{50} values had been set to one

Cell line	Actinomycin D	Colchicine	Vincristine	Daunorubicin
DC-3F	1	1	1	1
pLK212S-17	11.2 ± 1.8	22.4 ± 0.8	27.6 ± 2.2	16.1 ± 0.6
pHβneo124	0.74 ± 0.01	0.46 ± 0.02	0.13 ± 0.01	1.2 ± 0.2
pHβneoCAA	22.1 ± 1.8	64.1 ± 2.4	70.1 ± 0.6	65.0 ± 6.6
pHβneo124 + pHβneoCAA	43.5 ± 5.2	99.3 ± 4.3	76.5 ± 3.4	74.8 ± 1.7

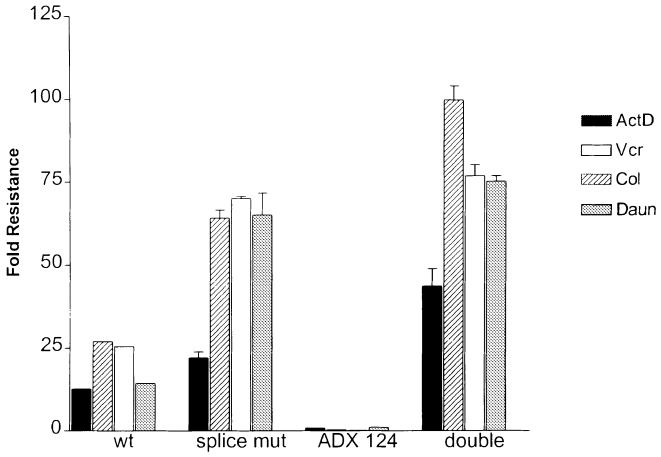

Fig. 3 Relative drug-resistant levels of transfectants. The data from Table 1 were used to construct the histogram, and the relative levels of resistance of each transfectant to the four drugs tested are shown (*wt* pLK212S-17, *splice mut* pHβneoCAA, ADX 124 pHβneo124, *double* cotransfectant)

The insert of pADX124 encodes a novel 57-kDa protein, $p57^{pgp1}$

When the transfectants described above were initially screened for pgp1 mRNA expression, they were also analyzed for the presence of P-gp by Western blot using the monoclonal antibody C219. The epitope for C219 is located near the C-terminal ATP binding fold of P-gp [28] and is retained in the putative protein encoded by the pADX124 cDNA insert (Fig. 1). However, as shown in Fig. 4, C219 did not detect the presence of a 57-kDa protein in any of the samples analyzed even though the 2.3-kb transcript was clearly expressed in both the pHβneo124 and pHβneoCAA transfectants (Fig. 2). A transfectant containing the pADX185 cDNA insert was also tested for expression and found to be negative (Fig. 3, lane 3). A second P-gp monoclonal antibody, C494 [28], the epitope for which is also present in the protein encoded by the pADX124 insert (Fig. 1) also failed to detect the presence of $p57^{pgp1}$ (data not shown). Any of three possibilities could account for these results: (1) the open reading frame present in the pADX124 insert is different from that present in pgp1, (2) the monoclonal antibodies C219 and C494 cannot recognize their corresponding linear epitopes within $p57^{pgp1}$ under

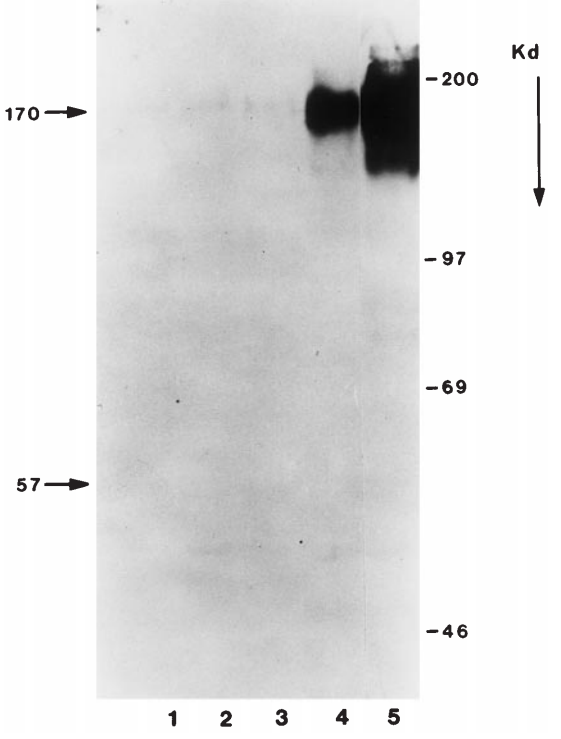

Fig. 4 Western blot analysis of transfectants. Cell lysate (50 μg) was electrophoresed on a 4.5 *M* urea/5% polyacrylamide gel, and transferred to a nitrocellulose membrane. P-gp was detected by monoclonal antibody C219 (Signet) using the ECL chemiluminescence system (DuPont NEN) (*lane 1* parental DC3F, *lane 2* pHβneo124 transfectant, *lane 3* pHβneo185, *lane 4* pHβneoCAA transfectant, *lane 5* pHβneo124-pHβneoCAA cotransfectant)

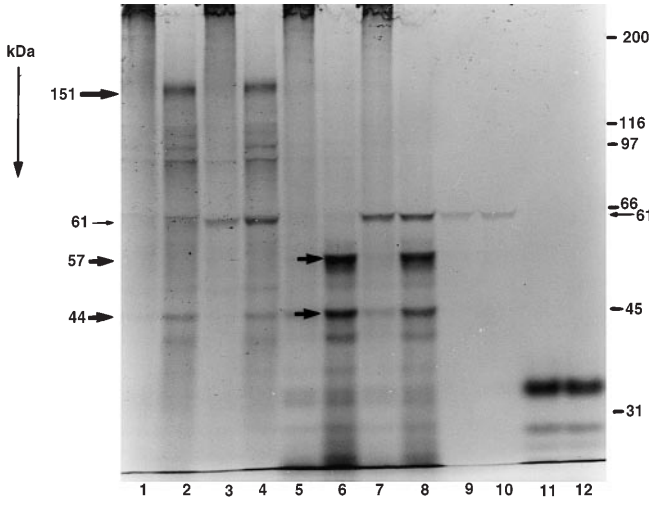

Fig. 5 In vitro translation of ADX124 and ADX165 mRNAs. Capped RNA (3 μg) was prepared by RiboMAX SP6 in vitro transcription and was added to an in vitro translation assay system using rabbit reticulocyte lysates supplemented with canine pancreas microsomal membranes. After a 60-min incubation at 30 °C in the presence of [^{35}S]-methionine, samples were either boiled or held at room temperature for 3 min and 25 μl of each then added to the wells of a 7.5% SDS polyacrylamide gel. Electrophoresis was at 22 V for 12 h (*lanes 1, 3, 5, 7, 9, and 11* contain boiled samples; *lanes 2, 4, 6, 8, 10, and 12* contain unboiled samples; *lanes 1 and 2* ADX165 RNA, *lanes 3 and 4* ADX165 RNA plus luciferase mRNA, *lanes 5 and 6* ADX124 RNA, *lanes 7 and 8* ADX124 RNA plus luciferase mRNA, *lanes 9 and 10* luciferase mRNA, *lanes 11 and 12* positive glycosylation controls supplied by the vendor). The *large horizontal arrow* indicates the position of pgp1, the *shorter arrows* the positions of $p57^{pgp1}$ and the 44 kDa peptide, and the *thin horizontal arrow* the position of the luciferase control peptide

the conditions used for Western analysis, or (3) the 2.3-kb transcript encoded by the pADX124 insert is not expressed as protein.

To address these issues, the cDNA inserts from pADX124 and pADX165 were cloned into pGEM4Z vectors in sense orientation to the Sp6 promoter and used as in vitro transcription templates for the synthesis of mRNA (see Materials and methods). These templates were then assayed for their ability to direct protein synthesis in vitro, in a rabbit reticulocyte lysate translation system supplemented with microsomes [29]. The results of these experiments are presented in Fig. 5 and show that both mRNAs were capable of directing the incorporation of [^{35}S]-methionine into peptides of molecular weights approximating those predicted from their encoded amino acid sequences. In the case of the pADX165 insert, the predicted molecular weight for the full-length amino acid backbone of the protein encoded by pgp1 is 140, 963 Da. The band shown in lane 2 of Fig. 4 is approximately 151 kDa. This difference in size has been shown previously to reflect the N-glycosylation of P-gp that occurs under experimental conditions very similar to the ones used here [30].

In the case of the pADX124 insert, the predicted molecular weight of the encoded protein is expected to be approximately 59 kDa. Two prominent [^{35}S]-methionine-labeled bands, one at approximately 57 kDa and another at 44 kDa are among the peptides encoded by this insert (Fig. 5, lane 6). Analysis of the insert's sequence indicates that there are at least four methionines that might serve as translational start codons. However, there are several reasons why it is most likely the first one at the 5′ end of the inferred mRNA sequence represents the translation initiation codon for p57^{pgp1}. First, it is the same start codon that is known to be used for translation of the full-length pgp1 mRNA [4], and its use would be consistent with the 'first AUG rule' [31]. Second, it maintains the open reading frame

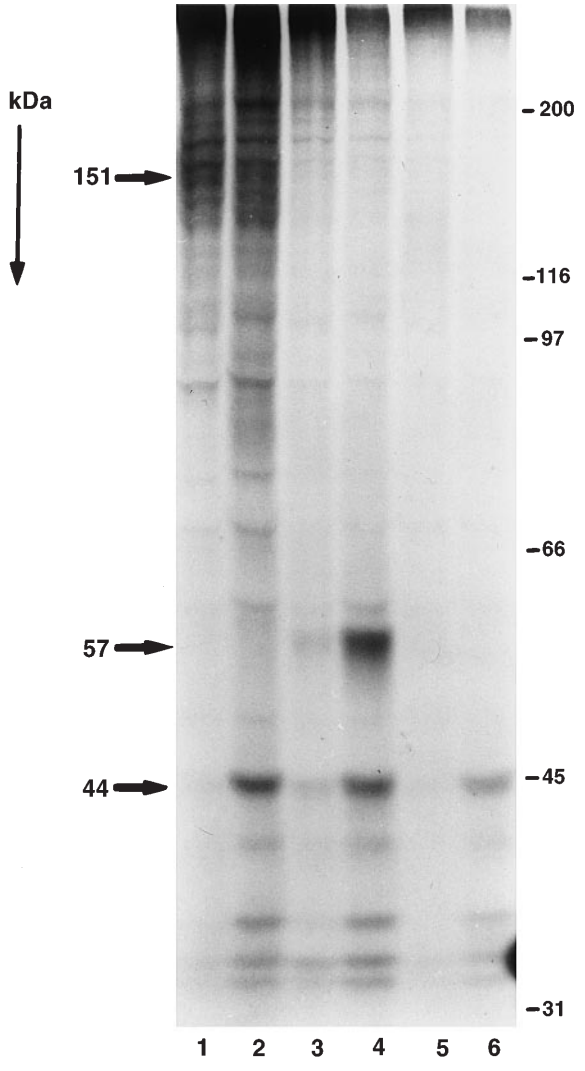

Fig. 6 In vitro translation in the presence or absence of microsomes. The in vitro translation conditions were as described in the legend for Fig. 5 except that the samples shown in *lanes 1, 3, and 5* were synthesized in the presence of microsomes, while those shown in *lanes 2, 4, and 6* were synthesized in the absence of microsomes. All samples were boiled for 3 min prior to loading the gel (*lanes 1 and 2* ADX165 RNA, *lanes 3 and 4* ADX124 RNA, *lanes 5 and 6* ADX185 RNA)

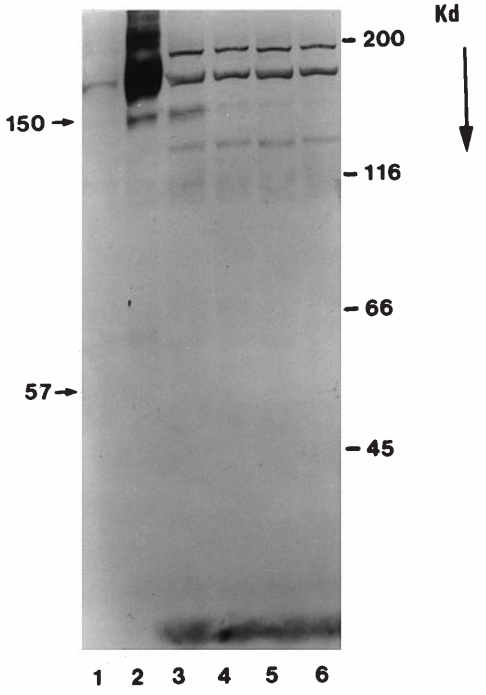

Fig. 7 Western blot analysis of in vitro translation products using monoclonal antibody C219. An in vitro translation assay similar to the one described in the legend for Fig. 5 was carried out, but with nonradioactive methionine. After electrophoresis, the gel was electroblotted onto nitrocellulose and analyzed by Western protocols using the P-gp-specific monoclonal antibody C219, as described in Material and methods (*lane 1* 50 μg total cell lysate from DC-3F cells, *lane 2* 50 μg total cell lysate from the pLK21S wild-type transfectant, *lane 3* ADX165 translation products, *lane 4* ADX124 translation products, *lane 5* luciferase RNA translation products, *lane 6* no-RNA control)

produced by the splicing reaction. Third, it is the only ATG codon in the 5′ end of the RNA encoded by the pADX124 insert located within a reasonable Kozak-like sequence context [32]. Moreover, it is the only one that can give rise to a peptide 57-kDa in size. These arguments strongly suggest that the [^{35}S]-methionine-labeled 57 kDa peptide is the one encoded by the pADX124 insert. The discrepancy in the size of the peptide between that predicted from the cDNA sequence and that observed from the SDS gel in Fig. 5 is most easily explained by the possibility that the peptide probably did not fully denature under the conditions used here for electrophoresis, and therefore did not migrate in direct proportion to its molecular size in this gel system.

The results shown in Fig. 5 also demonstrate that, like pgp1, p57^{pgp1} could not be denatured by boiling prior to electrophoresis because such treatment caused it to form large complexes that did not enter the gel (Fig. 5, lanes 1, 3, 5, and 7). The luciferase control peptide was not subject to such complex formation (Fig. 5, lanes 3, 4, 7, 8, 9, and 10) suggesting that these translation products differ greatly in their sensitivity to heat denaturation. However, all of the translations shown in Fig. 5 were done in the presence of microsomes, and it remained possible that this complex formation was dependent upon the presence of the microsomal fraction. We, therefore, carried out translations in the absence of microsomes and the results are presented in Fig. 6. All of the samples were subjected to boiling before electrophoresis, but microsomes were present only in the samples shown in Fig. 6, lanes 1, 3 and 5. As shown in lane 4, in vitro translation of the 2.3-kb RNA did not require the presence of microsomes and when boiled in their absence no complex formation was observed. Figure 6, lane 3 shows that when heated in the presence of microsomes, most of the p57^{pgp1} was complexed and did not enter the gel. Importantly, whether synthesized in the presence of microsomes (Fig. 5, lanes 6 and 8), or in their absence (Fig. 6, lane 4), the mobility of p57^{pgp1} did not change, indicating that this pgp1-related protein is not glycosylated by microsomal membranes in vitro.

Interestingly, the 44-kDa band present in Fig. 6, lane 4 was also present in lanes 2, 3, and 6. As predicted from the sequence of the 2.3-kb RNA, this peptide is likely to result from translation initiation at an internal Met codon. The presence of the 44-kDa peptide among the translation products generated from the RNAs obtained from the inserts of both pADX165 and pADX185, supports this interpretation and suggests that it is nonspecific in nature, while the absence of p57^{pgp1} from Fig. 6, lanes 2 and 6 argues for its specific translation from the 2.3-kb mRNA obtained from the pADX124 insert.

Taken together, these results show that p57^{pgp1} is a specific translation product of the 2.3-kb RNA transcript that does not require microsomes for synthesis in vitro and is not glycosylated. When heat-denatured in the presence of microsomes, its behavior mimics that of pgp1. The large molecular weight complexes that form under such conditions indicate that while p57^{pgp1} does not require microsomes for translation in vitro it may, nevertheless, be associated with them during or possibly after translation is complete.

To determine whether p57^{pgp1} was recognized by the monoclonal antibodies C219 and C494, we subjected samples of the above translation reactions carried out in the presence of membranes to Western blot analysis. As shown in Fig. 7, C219 was clearly able to recognize the full-length 150-kDa pgp1 encoded by pADX165 (lane 3), but was not able to recognize p57^{pgp1} (lane 4). Results with C494 were similar, although in some blots a faint, but irregular signal was observed coincident with the location of p57^{pgp1} (data not shown). These results suggest that, while the linear epitopes for both C219 and C494 are very likely to be present in p57^{pgp1}, they are apparently not accessible to these antibodies under the conditions used here for Western blot analysis.

p57^{pgp1} is expressed in vivo as a GFP fusion protein

Because neither C219 nor C494 was capable of recognizing p57^{pgp1}, we used a GFP fusion construct in which p57^{pgp1} provided the N-terminus to demonstrate the expression of this pgp1 splicing product in vivo. In this configuration, the expression of GFP requires the expression of p57^{pgp1} (see Materials and methods). The results of these experiments are shown in Fig. 8. In each of the four panels (Fig. 8A–D) approximately 30 cells are present in the field of view. Figure 8D shows a phase contrast image of the cells present in Fig. 8C and is typical of the fields present in Fig. 8A and B as well (not shown). The fluorescent cells in Fig. 8A show that the GFP vector used in these studies readily allowed the expression of GFP in CHL cells and that the protein was distributed nonspecifically throughout the cell. Figure 8B is a negative control in which the pADX124 insert was cloned in reverse orientation to GFP, preventing its expression, as indicated by the lack of fluorescence. Figure 8C shows that when the insert was cloned in-frame with GFP, the fusion protein was expressed as shown by the fluorescent signals. This result is consistent with the in vitro translation results presented above (Figs. 5 and 6) indicating that the pADX124 insert encodes a functional mRNA and shows that this mRNA is expressed in vivo. It is notable that the pattern of the fusion protein's expression is markedly different than that of GFP alone (compare Fig. 8A and C). The fusion protein appeared to be localized within organellar structures found in several cases to be adjacent to the nucleus. This can be readily observed by comparing the signals in Fig. 8C with the phase contrast image in Fig. 8D. The nature of these structures is not known.

Discussion

Overexpression of pgp1 mRNA by Chinese hamster lung tumor cells selected for MDR is accompanied by

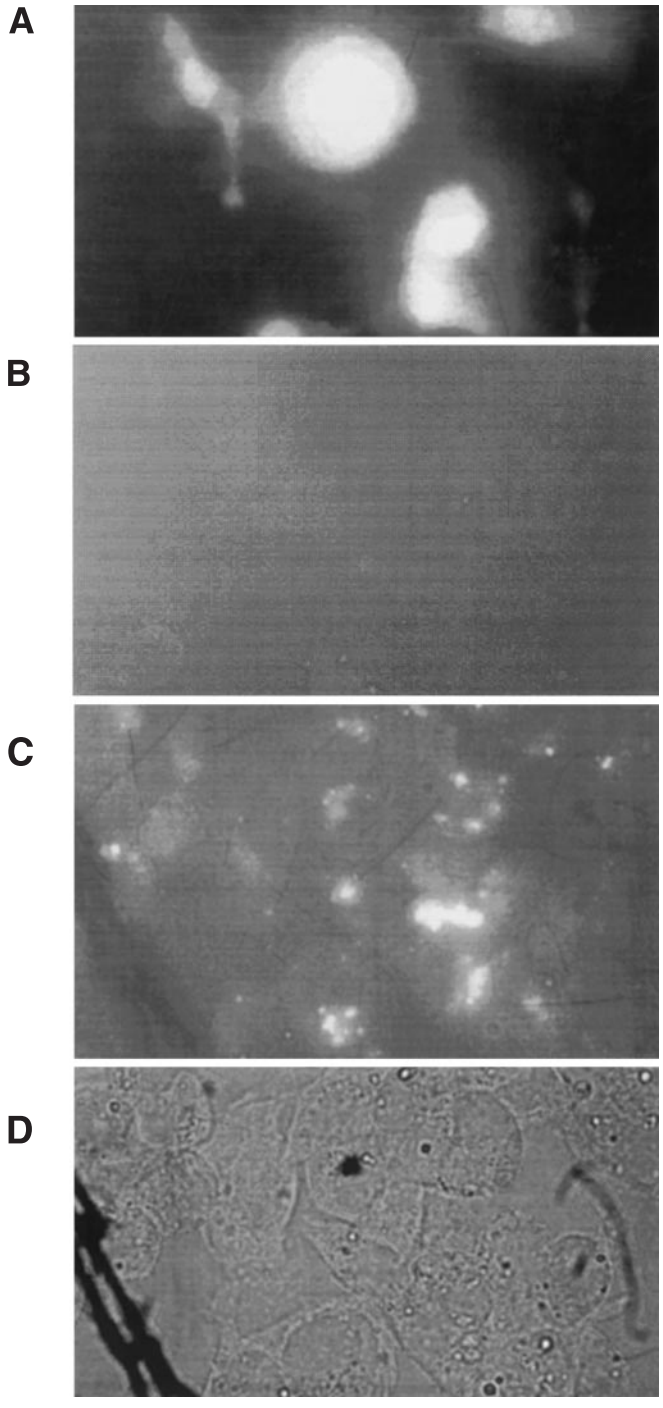

Fig. 8 Transient expression of a p57pgp1-GFP fusion protein in vivo. DC-3F cells were transfected with GFP fusion constructs as described in Materials and methods. After 72 h, cells were fixed with paraformaldehyde and analyzed by fluorescence microscopy. The digitized images shown were obtained by scanning the original photomicrographs with a Hewlett Packard Scan Jet 6100C flat-bed scanner and printing with Hewlett Packard Inkjet 1600 C on HP Premium Glossy Paper. Each field of view contains approximately 30 cells at a magnification of 100×. At least 200 cells were analyzed for each experiment (**A** GFP-positive control, B pADX124 cDNA insert cloned in reverse orientation to GFP, C pADX124 cDNA insert cloned in frame with GFP, D phase contrast photomicrograph of C)

overexpression of a smaller pgp1-related transcript [33]. A small transcript of similar size is also overexpressed when drug-sensitive cells are transfected with a full-length pgp1 cDNA [25]. In the studies reported here we attempted to investigate the potential significance of this smaller RNA. Isolation and nucleotide sequence analysis of pADX124, a clone obtained from a cDNA library of the MDR cell line DC-3F/ADX, indicated that its insert originated as a splicing product of the pgp1 gene [4]. The insert is 2.1 kb in length, and its 5′ leader begins 71 bases downstream of the +1 transcription start site of the pgp1 gene [34]. While it contains the polyadenylation signal known to be used for the full-length pgp1 transcript, its 3′ end terminates 15 nucleotides short of the polyadenylation site and it does not have a poly(A) tail [4]. Hence, in relation to its equivalent endogenous RNA transcript, the ADX124 cDNA is truncated by at least 85 nucleotides not including the length of its putative poly(A) sequence. This discrepancy would reasonably explain the difference in size noted between the 2.3-kb transcript and the pADX124 cDNA insert. We conclude, therefore, that the pADX124 cDNA insert does in fact represent the 2.3-kb transcript observed in selected MDR cell lines [33], and in transfectants expressing the pADX165 pgp1 cDNA from whose primary transcript it is derived by splicing (Fig. 2, lane 1).

When translated in vitro in a rabbit reticulocyte lysate system containing dog pancreas microsomes, the RNA transcript encoded by the pADX124 insert directed the synthesis of a 57-kDa peptide whose size was consistent with the most likely reading frame of the RNA (Fig. 5). Given that the reading frame is the same as that of pgp1, the epitopes for the monoclonal antibodies C219 and C494 should be present in $p57^{pgp1}$, and it is not clear why neither of these reagents identified this protein in vitro (Fig. 7) or in vivo (Fig. 4). Nor did these reagents recognize the 44-kDa peptide that results from initiation of translation at an internal Met codon in the 2.3-kb RNA and that, by sequence analysis, would also be expected to contain these epitopes. Since $p57^{pgp1}$ is clearly translated in vitro, and there is no obvious alternate reading frame of sufficient length within the pADX124 insert to generate a 57-kDa peptide, it is reasonable to conclude that the C219 and C494 epitopes are present in $p57^{pgp1}$ but are not recognized by these antibodies in Western blots. That this is likely due to structural inaccessibility caused by conformational differences between P-gp and $p57^{pgp1}$, is supported by the data presented in Fig. 8 showing that $p57^{pgp1}$ was readily expressed in transfectants as the N-terminal segment of a fusion product with GFP. Hence, p57pgp1 is expressed in vivo.

Previous S1 nuclease analysis has indicated that the 2.3-kb transcript, that we have now shown to be $p57^{pgp1}$ mRNA (Fig. 5), is present in all CHL cells that express pgp1 [4]. Splicing signals similar to those in hamster are also present in both mouse and human homologs of pgp1 [4]. It is not surprising, therefore, that the presence of pgp1-related transcripts of a size equivalent to the 2.3-

kb RNA in CHL cells have been reported in a number of MDR mouse and human cell lines [17–22]. There is some indication in human and mouse cells of hematopoietic origin, selected for resistance to either vincristine [20, 23] or adriamycin [21], that the relative level of expression of the smaller transcripts to the full-length transcripts is greater than it is in rodent fibroblasts [17, 18, 33]. While it is likely that homologs of $p57^{pgp1}$ mRNA are present in all cells that express class I P-gp, it is not clear whether they may be differentially expressed in MDR cell lines that have been established by selection. It is clear, however, that the level of expression of the small transcript is considerably higher in transfectants [25] than it is in selectants [33]. This most likely reflects the fact that the 4.3-kb primary transcript synthesized from the cDNA contains a single potential intron, whose splicing signals are more likely to be efficiently recognized as a pair when they are in relatively close proximity to one another. This is the case with the pADX124 cDNA where these signals are approximately 2.0 kb apart, as opposed to the full-length primary transcript where, based upon the mouse genomic sequence, they are more than 45 kb apart [35]. It is interesting to point out, however, that the 2.3-kb transcript is present in drug-sensitive parental DC-3F cells [4], and may be expressed as part of the default splicing pattern of the pgp1 gene. Its presence, therefore, may be of functional significance.

Our major interest is in the isolation and characterization of natural mutants of pgp1 that emerge during selection. Since the assay system for the study of such mutants relies upon transfection, it was of importance to define the effect that the 2.3-kb pgp1-splicing variant might have on the drug resistance patterns displayed by transfected cells. As indicated in Fig. 3 and in Table 1, elimination of the 2.3-kb transcript in transfectants that express full-length pgp1 mRNA did not dramatically alter cross-resistance, nor did the presence of the 2.3-kb transcript alone confer resistance. However, the presence of the 2.3-kb transcript did appear to decrease the level of resistance conferred by pgp1 expression, and this may help to explain why it is often difficult to predict drug resistance levels in cells based solely upon the amount of pgp1 mRNA expressed.

We have shown that the 2.3-kb mRNA can be translated in vivo as a component of a fusion construct with GFP (Fig. 8). It is likely, therefore, that $p57^{pgp1}$ per se is expressed in vivo as well, and it is interesting to speculate that the presence of this protein may in some way affect the efficiency of the pgp1 transporter. This either could occur by interfering with P-gp's ability to properly insert into the plasma membrane or perhaps by altering its interactions with other membrane proteins. Alternatively $p57^{pgp1}$ may affect the extent to which P-gp may form multimeric structures, or it may be involved in relocation of P-gp to sites other than the plasma membrane. In this regard it is interesting to note that whereas GFP was found uniformly distributed throughout the cell (Fig. 8A), the peptide made from $p57^{pgp1}$-GFP fusion constructs was found in what appeared to be intracellular structures (Fig. 8C). That the fusion protein is not located in the plasma membrane could be of functional significance. However, since this protein may not fold properly, it may simply be targeted for degradation via a lysosomal pathway. Studies in progress using a newly developed p57pgp1 polyclonal antibody (Staelens and Melera, in preparation) are attempting to determine the intercellular location of both endogenous and transfected $p57^{pgp1}$.

It is also not clear how $p57^{pgp1}$ may be related to a 65-kDa "mini-P-gp" ($P\text{-}gp_{mini}$) reported to be expressed in P388 murine leukemia cells selected for resistance to vincristine [22]. Western analysis using C219 as a probe indicated that P-gp is overexpressed in these cells, as is a 65-kDa peptide not present in controls. Using a human *MDR1* cDNA probe these authors also reported that in addition to full-length P-gp mRNA a 2.4-kb transcript was also overexpressed. However, no evidence was presented to suggest that the 2.4-kb transcript encoded the 65-kDa peptide. Given the fact that $p57^{pgp1}$ is not recognized by C219 and $P\text{-}gp_{mini}$ is, the two proteins are likely to be considerably different. However, the nucleotide sequence conservation between mouse and hamster pgp1 mRNA homologs is very high and the same splicing signals are present in both [4]. It is likely therefore, that the 2.4-kb transcript observed by these authors is the mouse equivalent of the 2.3-kb transcript we report here. It is also likely, given the results shown in Figs. 3 and 7, that if the mouse equivalent of $p57^{pgp1}$ were expressed in P388/VCR600 cells, it would not have been identified by C219. Hence, the nature and the origin of "$P\text{-}gp_{mini}$" remain to be defined.

Finally, the results presented here suggest that the expression of all pgp1 transgenes may be mediated by splicing, and that the level of pgp1 mRNA in transfectants is likely to be lower than would be expected based upon promoter efficiencies. We estimate that as much as 40–50% of the full-length pgp1 transcripts expressed from the pHβneo vectors used here are converted to the 2.3-kb transcript (Fig. 2; [25]). Given the likelihood that these RNAs are also translated into proteins it will be of interest to determine what other aspects of the "multidrug resistance phenotype" [33] they affect.

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