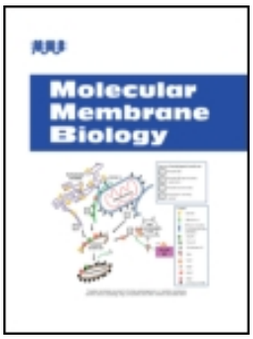

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Mutational analysis of the major proline transporter (PrnB) of *Aspergillus nidulans*

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Summary

PrnB, the L-proline transporter of *Aspergillus nidulans*, belongs to the Amino acid Polyamine Organocation (APC) transporter family conserved in prokaryotes and eukaryotes. *In silico* analysis and limited biochemical evidence suggest that APC transporters comprise 12 transmembrane segments (TMS) connected with relatively short hydrophilic loops (L). However, very little is known on the structure-function relationships in APC transporters. This work makes use of the *A. nidulans* PrnB transporter to address structure-function relationships by selecting, constructing and analysing several *prnB* mutations. In the sample, most isolated missense mutations affecting PrnB function map in the borders of cytoplasmic loops with transmembrane domains. These are I119N and G120W in L2-TMS3, F278V in L6-TMS7, NRT378NRTNRT and PY382PYPY in L8-TMS9 and T456N in L10-TMS11. A single mutation (G403E) causing, however, a very weak phenotype, maps in the borders of an extracellular loop (L9-TMS10). An important role of helix TMS6 for proline binding and transport is supported by mutations K245L and, especially, F248L that clearly affect PrnB uptake kinetics. The critical role of these residues in proline binding and transport is further shown by constructing and analysing isogenic strains expressing selected *prnB* alleles fused to the gene encoding the Green Fluorescent Protein (GFP). It is shown that, while some *prnB* mutations affect proper translocation of PrnB in the membrane, at least two mutants, K245E and F248L, exhibit physiological cellular expression of PrnB and, thus, the corresponding mutations can be classified as mutations directly affecting proline binding and/or transport. Finally, comparison of these results with analogous studies strengthens conclusions concerning amino acid residues critical for function in APC transporters.

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Keywords: *Aspergillus nidulans*, proline, transport, mutagenesis, structure-function analysis.

Abbreviations: TMS, transmembrane segment, L, hydrophilic loop, CAR, Consensus Amphipathic Region, APC, Amino acid Polyamine Organocation transporter family, GFP, Green Fluorescent Protein, MM and CM, minimal and complete media respectively, K_m, Michaelis constant, V_{max}, maximum velocity of transport.

Introduction

Amino acids and their derivatives are transported into and out of cells by a variety of transporters which comprise several distinct protein families, some of which are distantly related (Saier 2000). The largest and best-studied amino acid transporter family is the Amino acid Polyamine Organocation (APC) transporter family (Sophianopoulou and Diallinas 1995, Saier 2000). The APC transporter superfamily includes members that function as solute/cation symporters and solute/solute anti-porters. They are found in bacteria, archaea, fungi, eukaryotic protists, plants and animals. They vary in length, from 350–850 residues. Most of them possess 12 transmembrane α-helical segments (TMS), but members of some sub-families might have 10, 11 or 14 TMS. One APC family member, Hip1p of *Saccharomyces cerevisiae* has been implicated in heavy metal transport (Farcasanu *et al*. 1998). Interestingly, three integral membrane receptors of mammals, the ecotropic retroviral leukaemia receptor (ERR), the human retroviral receptor (HRR) and the T-cell early activator (T_{ea}), are homologous to APC transporters (Reizer *et al*. 1993). The ERR protein has been shown to function as a cation/amino acid co-transporter (Wang *et al*. 1991). Other proteins, including the developmentally controlled GerAII spore germination protein of *Bacillus subtilis* and the acetylcholine receptor of *Drosophila melanogaster* might also share a common evolutionary origin with members of the APC family (Reizer *et al*. 1993).

Several APC bacterial and fungal amino acid transporters have been identified and studied in great detail at the level of transport kinetics and regulation of expression (Sophianopoulou and Diallinas 1995, Saier 2000, Burkovski and Kramer 2002 and references therein). Fungal and bacterial amino acid transporters show significant sequence similarities (33–62% identity scores in binary comparisons) that may reflect a common topology and mechanism of action. Their specificities range from one to several L-amino acids and their kinetics of transport and regulation of expression may also vary significantly (Sophianopoulou and Diallinas 1995, Saier 2000). Studies addressing how yeast amino acid transporters find their way to the plasma membrane have shown that their topogenesis depends on both general and family-specific secretion factors or chaperones (Martinez and

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Ljungdahl 2000). Finally, recent studies have shown that homologues of amino acid transporters in yeast function as 'sensors' of amino acids rather than real transporters (Bernard and André 2001, Forsberg and Ljungdahl 2001).

In *Aspergillus nidulans*, the *prnB* gene (Sophianopoulou and Scazzocchio 1989) encodes a highly specific transporter for L-proline (Sophianopoulou and Diallinas 1995, Tazebay *et al*. 1995). The *prnB* gene is located in the *prn* gene cluster encoding all proteins necessary for proline catabolism (Hull *et al*. 1989). Expression of the *prnB* gene has been studied in great detail and shown to be induced by proline, amino acid starvation and conidial germination and to be repressed by the simultaneous presence of ammonia and glucose (Arst and Cove 1973, Sophianopoulou *et al*. 1993, Tazebay *et al*. 1995, Gonzalez *et al*. 1997, Tazebay *et al*. 1997, Cubero *et al*. 2000).

This work makes use of the *A. nidulans* PrnB transporter to address structure-function relationships by selecting, constructing and analysing several *prnB* mutations. Studying chimeric proteins carrying PrnB mutations fused to the green fluorescent protein (GFP) allowed one to classify several mutations to those affecting PrnB topogenesis and to those directly affecting proline binding and transport. The results also showed that several of the amino acids affecting PrnB function are located in similar regions with residues affecting the function of homologous APC transporters from bacteria, yeast and mammals.

Results and discussion

Genetic isolation of prnB mutations

Early genetic work has led to the isolation of several *prnB*-specific loss-of-function mutations (*prnB6*, *prnB32*, *prnB36*, *prnB81*, *prnB82*, *prnB1110*, *prnB206*; Arst and MacDonald 1975, Arst *et al*. 1981, Jones *et al*. 1981, Arst Jr., H. N., personal communication). A detailed genetic map including most of these mutations has been constructed and correlated with the physical map available for the *prnB* locus (Durrens *et al*. 1986). To enlarge the original collection of *prnB* mutations and gain further insights into the process of PrnB structure and function, the original genetic selection was modified in order to isolate partial loss-of-function or cryosensitive mutations (see Experimental procedures). Such mutations are more likely to affect PrnB function rather than its expression. One, thus, isolated six novel *prnB* mutations called *prnB115*, *prnB117*, *prnB119*, *prnB144*, *prnB411* and *prnB508*.

All *prnB* mutants isolated previously and herein were analysed by simple growth tests for their ability to grow on proline or other nitrogen sources. Figure 1 shows selected results for growth on proline at 25 and 37°C. Within the limits of growth tests, two types of mutants could be distinguished. Type 1 mutants, which included all the unconditional mutations from the original collection of Arst *et al*. (1981), showed a growth phenotype identical to that of the total loss-of-function mutation *prnB377* resulting from an internal deletion in the *prnB* gene (Tazebay *et al*. 1995, 1997). Type 2 mutants (*prnB115*, *prnB119*, *prnB144*, *prnB411*, *prnB117* and *prnB508*) showed a growth phenotype compatible, albeit at different degrees, with a partial loss of PrnB function, more evident at 25°C than at 37°C. None of the mutations resulted in a pH-dependent phenotype and none affected growth on other nitrogen sources tested (results not shown).

Sequence changes of prnB mutations

The entire *prnB* open reading frame of each of the mutants described above was sequenced. The region between the start and stop codons of *prnB* was amplified by the polymerase chain (PCR) reaction, as described in Experimental procedures. PCR products were either sequenced directly or cloned into bluescript KS(+) and sequenced using a series of *prnB*-specific oligonucleotides (see Experimental procedures). Unique mutations were found in every case. The substitutions identified are summarized in Table 1. Among the 13 mutations sequenced, four (*prnB32*, *prnB36*, *prnB82* and *prnB1110*) corresponded to nonsense and/or frameshift mutations and were not analysed any further in this work. The physical location of the mutations that have been mapped previously (Jones *et al*. 1981) is completely congruent with their location in the genetic fine structure map (results not shown, Durrens *et al*. 1986). All isolated mutations studied further were renamed on the basis of the corresponding amino acid substitution (see Table 1).

A mutation affecting prnB mRNA steady state levels

To rule out the possibility that isolated mutations affect *prnB* expression rather than PrnB function, it was investigated whether basal *prnB* mRNA steady state levels are comparable to wild-type levels. Northern blot analyses were carried out of all *prnB* mutations at 25 and 37°C. It was found that all mutant strains, except *prnB-Extended* (*prnB115*), showed mRNA levels identical to those of a *prnB*$^+$ strain at both temperatures (results not shown). Mutant *prnB-Extended* (*prnB115*) showed reduced *prnB* mRNA at 25°C (Figure 2(*a*)). This mutation is due to a frameshift mutation in the physiological stop codon of the *prnB* gene, resulting in a seven amino acids extension of the PrnB open reading frame. Interestingly, the region of the *prnB* mRNA immediately downstream from the stop codon contains sequences which might form a stem-loop structure (Figure 2(*b*)). In some cases, such structures have been shown to be critical for mRNA stability and turnover (Causton *et al*. 1994, Platt *et al*. 1996). Thus, one can speculate that extended translation of this region might well disrupt the formation of this stem-loop secondary structure and, thus, affect *prnB* mRNA steady state levels.

Design and construction of site-directed prnB mutations

A second approach to identify residues important for PrnB function or specificity was to construct by *in vitro* directed mutagenesis missense mutations altering selected amino acids conserved in the proline transporters of *A. nidulans* (PrnB) and *Saccharomyces cerevisiae* (Put4p). The mutations introduced concern two specific amino acid residues, Q219 located in L5 and K245 located within TMS6 of the

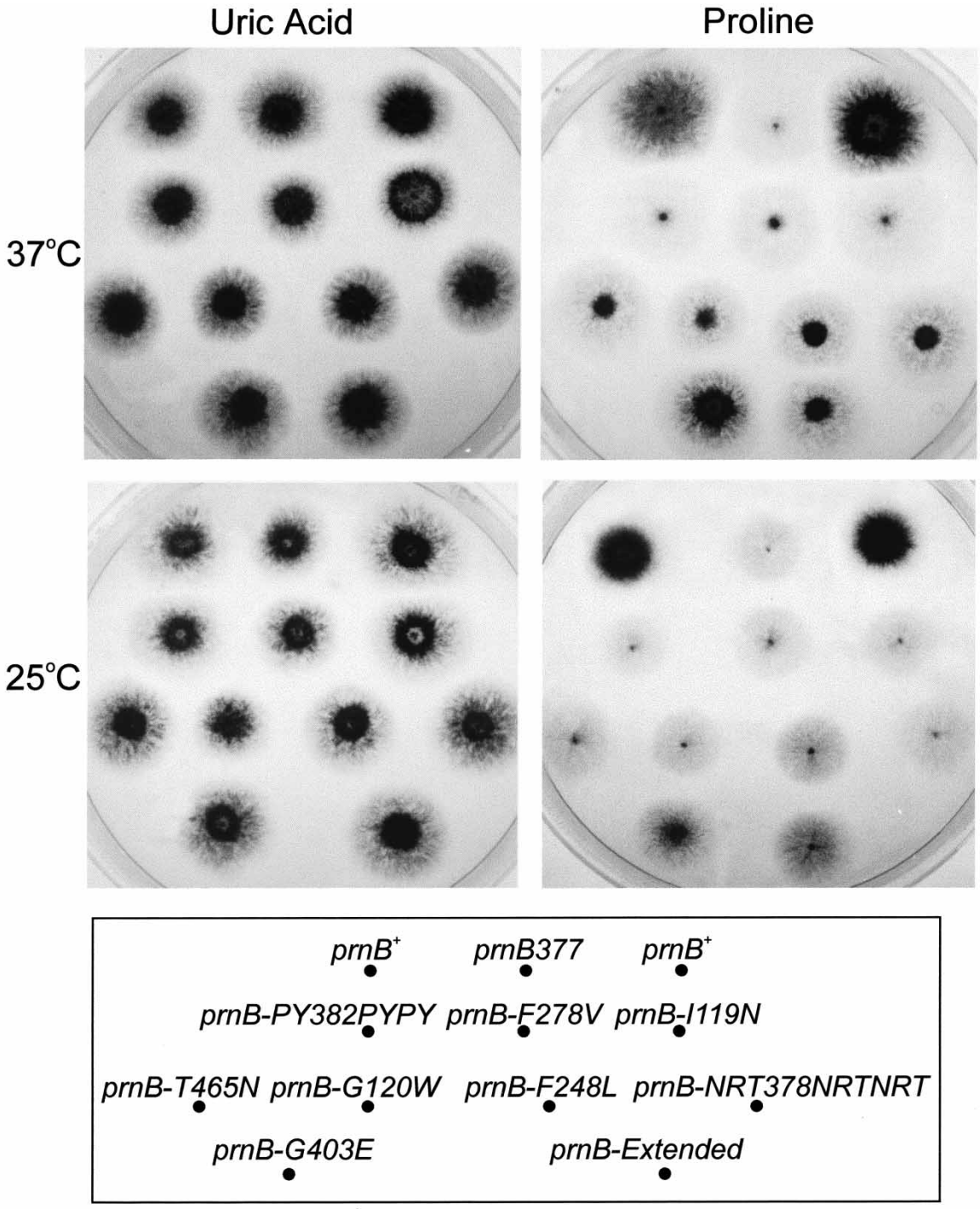


Figure 1. Growth of *A. nidulans* mutant strains. Growth of control strains (*prnB*$^{+}$, *prnB377*) and mutant strains (*prnB-PY382PYPY*, *prnB-F278V*, prnB-*I119N*, *prnB-T456N*, *prnB-G120W*, *prnB-F248L*, *prnB-NRT378NRTNRT*, *prnB-G403E* and *prnB-Extended*). Because *prnB* mutations exist in either green (*yA*$^{+}$) or yellow (*yA2*) conidiospore genetic background, the corresponding *prnB*$^{+}$ strains are shown. Growth tests were carried out on *A. nidulans* minimal medium supplemented with 595 μM uric acid or 5 mM proline as sole nitrogen sources at 25 and 37°C, as indicated. Growth tests were carried out for 60 h at 25°C or 48 h at 37°C.

PrnB protein. The choice was based on a number of observations. Both residues are located in segments of high conservation in fungal transporters (Figure 3). In addition, K245 is one of the two positively charged residues located within a TMS of PrnB. The other is H334 located within TMS8. Interestingly, a Lys residue in TMS5 of the phenylalanine transporter (PheP) of *E. coli* has been shown to be critical for transport activity (Pi *et al*. 1993). Charged or polar amino acid residues in general might be involved directly or indirectly in protein function.

Table 1. Mutant alleles of *prnB* and their phenotypes on proline as sole nitrogen source.

prnB allele	Phenotype on proline 25°C	37°C	Nucleotide change	Protein change	L-TMS
prnB^{+}	++++	++++	–	–	–
prnB377	–	–	Deletion (Tazebay *et al*. 1995)	Deletion of 249 amino acids	Deletion of TMS6-TMS11
prnB6	–	–	$ATT^{357} \rightarrow AAT^{357}$	PrnB-I119N	L2-TMS3
PrnB32	–	–	$TGG^{1328} \rightarrow TGA^{1328}$	PrnB-W409stop	L9-TMS10
prnB36	–	–	$TGG^{1136} \rightarrow TGA^{1136}$	PrnB-W345stop	TMS8
prnB81	–	–	$TTC^{935} \rightarrow GTC^{935}$	PrnB-F278V	L6-TMS7
prnB82	–	–	Insertion G^{1093}	PrnB-Frameshiftstop (TAA^{1148})	TMS8
prnB206	–	–	Duplication $CCCTAT^{1247}$	PrnB-PY382PYPY	L8-TMS9
prnB1110	–	–	$TAT^{959} \rightarrow TAA^{959}$	PrnB-Y286stop	TMS7
prnB144	–	++	$GGG^{360} \rightarrow TGG^{360}$	PrnB-G120W	L2
prnB411	–	++	$ACC^{1517} \rightarrow AAC^{1517}$	PrnB-T456N	L10-TMS11
prnB115	+(+)	++	Insertion T^{1801}	PrnB-Extended	C-term
prnB117	–	++	Duplication $CCAACCGCA^{1233}$	PrnB-NRT378NRTNRT	L8-TMS9
prnB119	+	++	$TTT^{845} \rightarrow TTG^{845}$	PrnB-F248L	TMS6
prnB508	++	+++	$GGA^{1310} \rightarrow GAA^{1310}$	PrnB-G403E	L9-TMS10

+++ define wild-type growth, – defines lack of growth typical of the non-utilization of a N source, and different number of + describe intermediate growth between +++ and –.

Mutations *prnB-Q219R*, *prnB-Q219H*, *prnB-K245R*, *prnB-K245E* and *prnB-K245L* were constructed and introduced by targeted homologous integration into the *prnB* genomic locus of *A. nidulans*, as described in Experimental procedures and previously in Tavoularis *et al*. (2001). Mutations *prnB-Q219R* and *prnB-Q219H* substitute the Gln found in proline transporters for two residues conserved in all other fungal amino acid transporters. Mutations *prnB-K245R*, *prnB-K245E* and *prnB-K245L* help define the role of the positive charge present in TMS6 of proline transporters. All mutant strains were analysed by growth tests for their ability to grow on proline or other nitrogen sources. Figure 4 shows that *prnB-Q219R*, *prnB-Q219H*, *prnB-K245L* and *prnB-K245R* mutant strains grow similarly to a *prnB*$^{+}$ strain in media containing proline as the sole nitrogen source at both 37 and 25°C. Mutant strain *prnB-K245E* showed a mildly reduced growth on proline at 25°C but normal growth at 37°C. All mutants showed normal growth on all other nitrogen sources tested (results not shown).

Transport properties of the prnB mutants

A kinetic analysis was carried out of proline uptake in all mutant strains described in this work. All uptake experiments were performed at 37°C. It should also be emphasized that *V*-values represent apparent capacities for proline transport, as they are directly dependent on the amount of PrnB present at the plasma membrane, a variable that one cannot

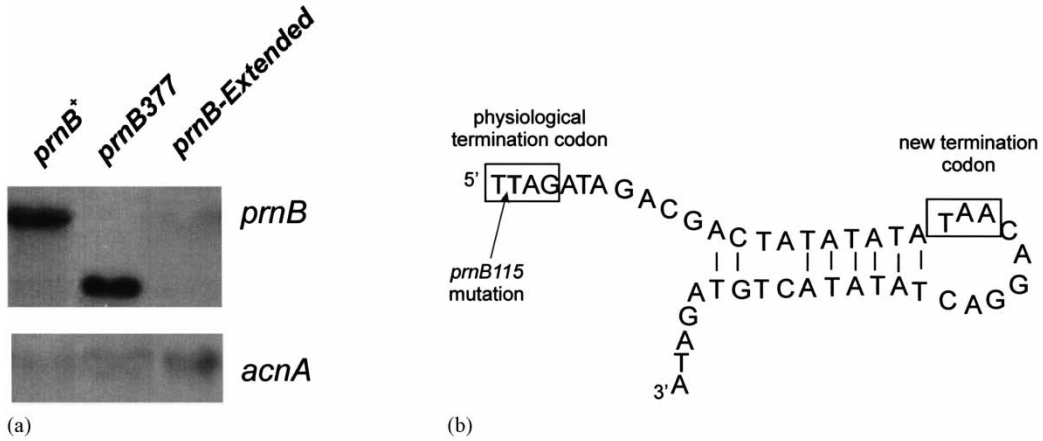


Figure 2. *prnB* mRNA steady state levels (*a*) and model of *prnB-Extended* stem-loop structure (*b*). (*a*) Northern blot analysis of 10 μg total RNA extracted from mycelium grown at 25°C for 16 h under non-inducing conditions (urea as nitrogen source) followed by 4 h of growth in the presence of 20 mM L-ornithine (inducing conditions independent of PrnB function; ornithine is taken up by a transporter other than PrnB and is converted intracellularly to proline (Dzikowska *et al.* 1999). Therefore, *prnB* mutations do not affect induction by ornithine). RNA was extracted from a *prnB*$^{+}$ strain, a strain carrying a *prnB* deletion (*prnB377*) and the mutant strain *prnB-Extended*. RNA transferred onto nitrocellulose filters was hybridized with *prnB*- and *acnA*-specific probes, as described in Experimental procedures. *acnA* is the *A. nidulans* actin gene used as an internal control to monitor RNA amounts (Fidel *et al.* 1988). (*b*) PrnB-Extended stem-loop structure. Analysis of the 3′ mRNA region of the *prnB-Extended* allele using the DNA Strider programme (Marck 1988). The stop codons of the *prnB*$^{+}$ and the *prnB-Extended* alleles are shown within boxes. The possible interactions for the formation of a secondary structure are indicated by vertical lines. The selection of the region analysed is arbitrary.

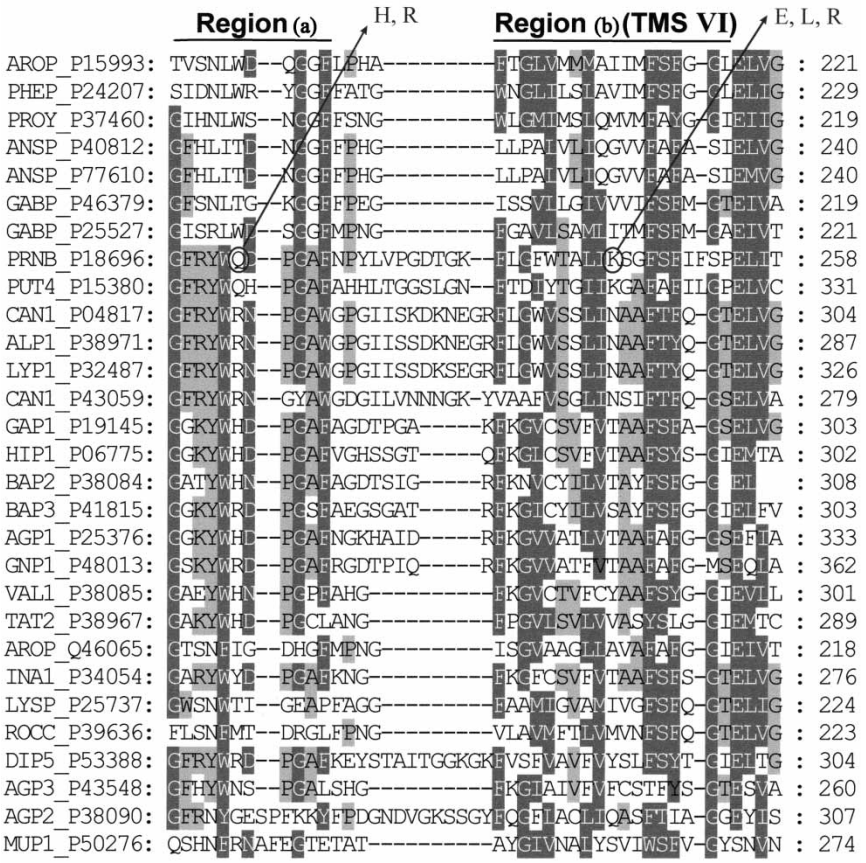


Figure 3. Sequence alignment of bacterial and fungal amino acid transporters. Two regions of high amino acid similarity, (*a*) and (*b*), are presented. Region (*a*) is located in L5 and region B in TMS6 in all amino acid transporters. Q219 and K245 (numbering refers to *A. nidulans*) conserved only in proline transporters of *A. nidulans* and *S. cerevisiae* map in regions (*a*) and (*b*), respectively. Similar amino acids are shown in dark and light grey boxes, according to the degree of similarity ($>60\%$ and $>40\%$, respectively). Small dashes indicate gaps introduced by the programme to maximize similarity. AROP (P15993), PHEP (P24207), ANSP (P77610), GABP (P25527) and LYSP (P25737) are *E. coli* permeases, specific for aromatic amino acids, phenylalanine, asparagine, GABA and lysine, respectively. PROY (P37460) and ANSP (P40812) are *Salmonella typhimurium* permeases, specific for proline and asparagine, respectively. GABP (P46379) and ROCC (P39636) are *B. subtilis* permeases, specific for GABA and possibly all amino acids, respectively. AROP (Q46065) is a permease specific for aromatic amino acids from *Corynebacterium glutamicum*. INA1 (P34054) is a putative general amino acid permease from *Trichoderma harzianum*. PRNB is the *A. nidulans* proline-specific permease. CAN1 (P43059) is a permease-specific for the basic amino acids lysine and arginine from *Candida albigans*. PUT4 (P15380), CAN1 (P04817), ALP1 (P38971), LYP1 (P32487), HIP1 (P06775), BAP2 (P38084), BAP3 (P41815), AGP1 (P25376), GNP1 (P48813), VAL1 (P38085), TAT2 (P38967), DIP5 (P53388) and MUP1 (P50276) are *S. cerevisiae* permeases specific for proline, arginine, basic amino acids, lysine, histidine, leucine/valine/isoleucine, valine, asparagine/glutamine, glutamine, valine/tyrosine/tryptophan, tryptophan, glutamate/aspartate and methionine, respectively. Finally, GAP1 (P19145), AGP3 (P43548) and AGP2 (P38090) are general amino acid permeases from *S. cerevisiae*. In parentheses, the accession number of each protein in the SwissProt database is indicated. The directed mutations carried out in the present work are indicated by arrows.

estimate immunologically due to very low PrnB expression levels (Tavoularis *et al*. 2001).

In agreement with growth tests, mutants not growing on proline as a sole nitrogen source (nonsense or frameshift mutations and missense mutations *prnB-I119N*, *prnB-F278V* and *prnB-PY382PYPY*) show the same level of residual proline uptake as that found in a strain carrying a deletion of the *prnB* gene (results not shown). This is due to other minor amino acid transporter(s) able to incorporate proline and it never exceeds 20% of the total uptake (Arst *et al.* 1980, Tazebay *et al*. 1995, Scazzocchio C. and Apostolaki A., Personal communication). Table 2 summarizes the results obtained with all other mutations studied. All partial loss-of-function mutants obtained by selection for proline toxicity (*prnB-NRT378NRTNRT*, *prnB-Extended*, *prnB-F248L*, *prnB-G120W*, *prnB-T456N* and *prnB-G403E*) have 1.5–5-fold reduced apparent *V*-values, in agreement with their reduced growth on proline as a nitrogen source (see Figure 1). Among these mutations, only *prnB-F248L* also reduces the affinity of PrnB for proline significantly (4–4.5-fold). Substitutions of Q219 had a significant (6-fold) up-effect on the capacity of PrnB to transport proline and have no obvious growth phenotype on proline as a nitrogen source. Different substitutions of K245 had different effects on PrnB function. Mutant *prnB-K245R* showed proline uptake kinetics nearly identical to a $prnB^+$ strain. While both *prnB-K245L* and *prnB-K245E* mutants showed approximately 2–3-fold reduced capacity for proline transport, the former has a

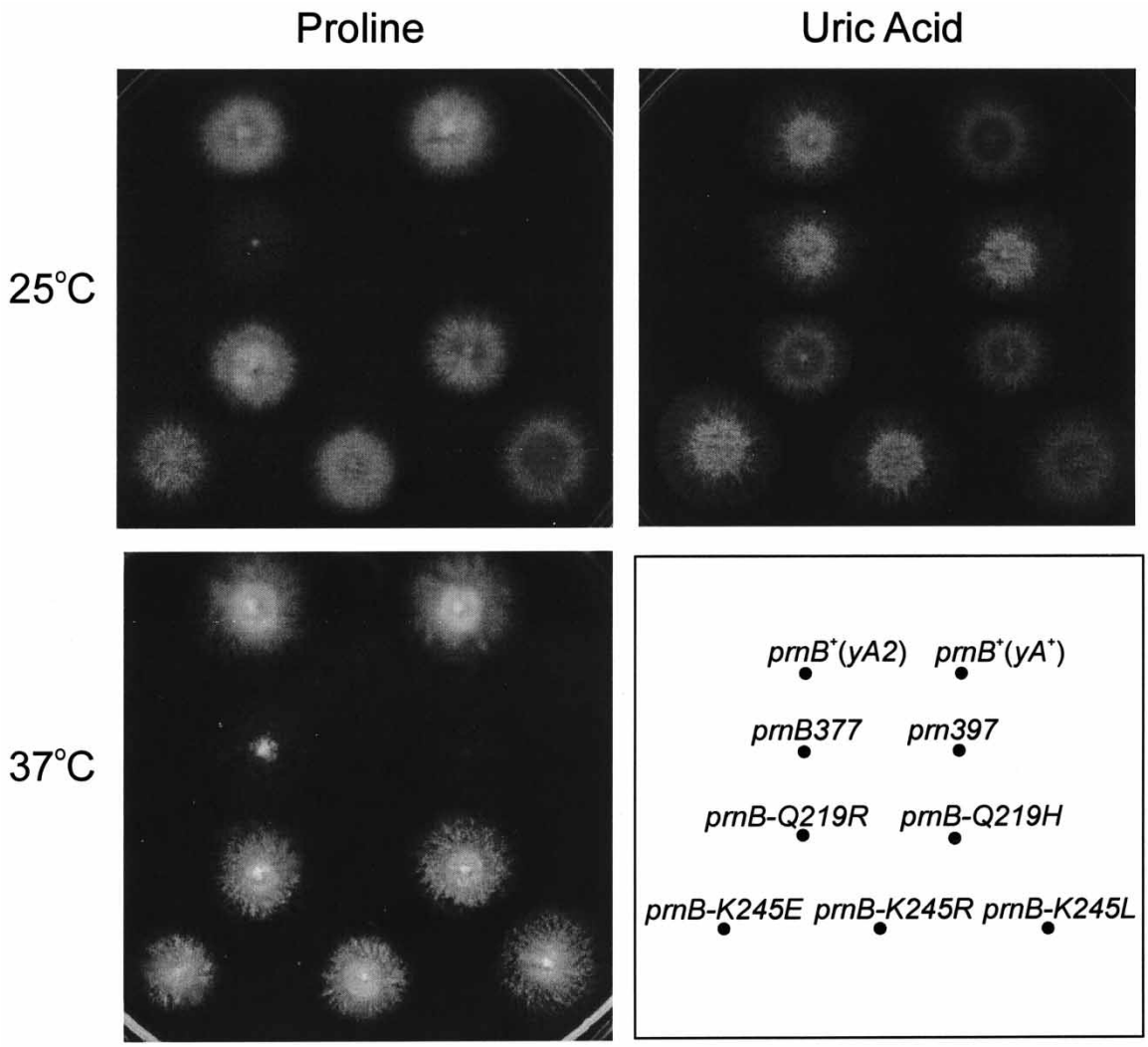


Figure 4. Growth of *A. nidulans* mutant strains. Growth of control strains (*prnB*$^+$, *prnB377*), the recipient strain *prn397* and the mutant strains (*prnB-Q219R*, *prnB-Q219H*, *prnB-K245E*, *prnB-K245R* and *prnB-K245L*) is shown. Because *prnB* mutations exist in either a green (*yA*$^+$) or yellow (*yA2*) conidiospore genetic background, the corresponding *prnB*$^+$ strains are shown. Growth was tested on *A. nidulans* MM supplemented with 5 mM proline as sole nitrogen sources at 25 and 37°C, as indicated. Strains grown on 595 μM uric acid as a nitrogen source at 37°C are also shown. Growth tests were carried out for 60 h at 25°C or 48 h at 37°C. Note the difference in growth between *prnB397* (deletion of *prnB* and *prnC*) and *prnB377* (deletion of *prnB*) strains, which allows recovery of even a complete loss-of-function mutation in *prnB* by the selection technique employed (see Experimental procedures).

significantly more reduced affinity for proline transport than the latter (8-fold compared to 2-fold). None of these changes affects growth of these mutants on proline (see Figure 4).

Interestingly, *prnB-F248L* and *prnB-K245L*, the mutations affecting most (4- and 8-fold, respectively) the affinity of PrnB for its substrate, concern two amino acid residues on the same side of the TMS6 α-helix (see below and Figure 6). F248 is an absolutely conserved residue in the APC family while K245 is conserved only in fungal proline transporters. One could speculate that such amino acids could directly or indirectly affect binding of substrates. Mutation *prnB-F248L* presented an additional interest due to work carried out in an homologous transporter. It has been shown that substitution of the homologous Phe residue for a Ser in Bap1p (branched chain amino acid transporter) and Hip1p (histidine transporter; Farcasanu *et al*. 1998) in *S. cerevisiae* led to transporters with reduced amino acid uptake capacity, resistant to low pH and with the novel ability to take up K^+ ions (Wright *et al*. 1997). It was examined whether the mutation *prnB-F248L* had a similar effect on PrnB function. Within the limits of growth tests carried out in the presence of different concentrations of KCl (10 and 100 mM) and different pHs (4.8, 6.8 and 9.0) and uptake studies in different concentrations of KCl (10 and 100 mM), one has been unable to demonstrate similar effects (results not shown). This might depend on which amino acid substitutes Phe.

One has also examined whether any of the mutations studied affect the absolute dependence, seen for the wild-type PrnB transporter (unpublished results), on a H^+ gradient across the plasma membrane. In all PrnB mutants, similar to a wild-type strain, pre-incubation with H^+-ATPase inhibitors (N′,N′-dicyclohexylcarbodiimide) or protonophores

Table 2. K_m and V-values for ^{3}H-proline uptake in the wild-type and in strains carrying PrnB mutations.

Strains	K_m (μM)	V_{max} (pmoles min^{-1} 10^8 viable conidiospores^{-1})
prnB$^+$	34 ± 2.0	8.0 ± 0.2
prnB-NRT378NRTNRT	50 ± 2.0	3.2 ± 0.05
PrnB-Extended	30 ± 2.0	6.2 ± 0.1
prnB-F248L	134 ± 10	4.5 ± 0.1
prnB-G120W	33 ± 2.0	1.5 ± 0.05
prnB-T456N	35 ± 2.0	1.8 ± 0.05
prnB-G403E	71 ± 3.0	4.0 ± 0.1
prnB-Q219R	74 ± 3.0	52.8 ± 3.0
prnB-Q219H	58 ± 3.0	43.5 ± 3.0
prnB-K245E	61 ± 3.0	2.9 ± 0.05
prnB-K245L	281 ± 10	4.3 ± 0.1
prnB-K245R	34 ± 2.0	6.7 ± 0.1

Note: Proline uptake assays were performed as described in Experimental procedures. Proline uptake is expressed in pmoles per minute per 10^8 viable conidiospores. The values shown represent averages of at least three independent assays showing no significant deviation.

(carbonyl cyanide *m*-chlorophenyl-hydrozone) abolished PrnB-dependent proline uptake (results not shown), indicating that PrnB is a proline/H^+ symporter.

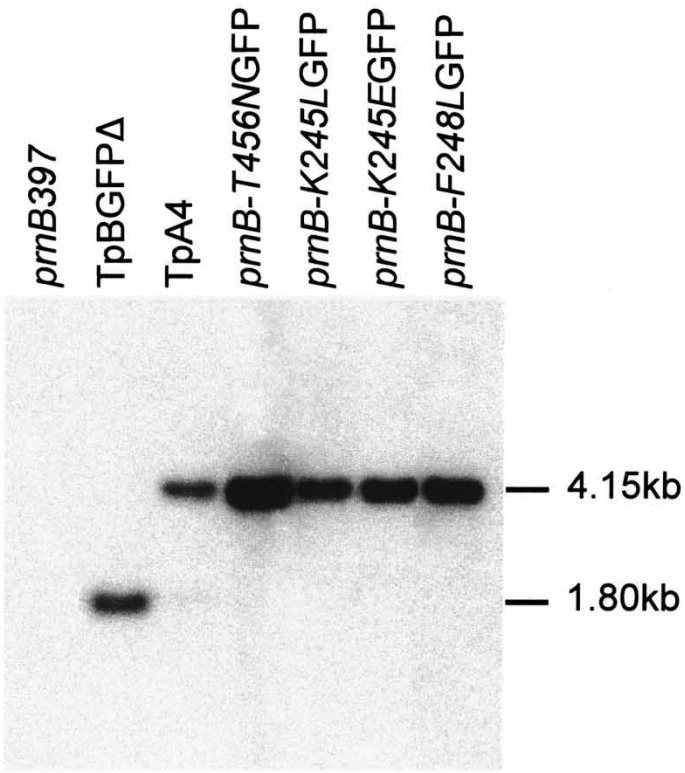
Cellular expression of PrnB in selected mutant

Results presented above clearly show that two mutations (K245L, F248L). affect the affinity (K_m) of PrnB for proline. However, as far as it concerned the effect of different mutations on proline transport (V), one could not draw any conclusion unless the amount of the protein in the plasma membrane was estimated. As has been discussed previously (Diallinas *et al*. 1998), the immunological detection of *Aspergillus* transporter proteins expressed from their native promoters has not been possible, and this is common also for many other transporters from different organisms, possibly due to low expression levels (Tavoularis *et al.* 2001 and references therein). On the other hand, over-expression of transporters often elicits a stress signal on the secretory pathway (unpublished observations) and induces the Unfolded Protein Response (Ma and Hendershot 2001). Thus, one has previously described the systematic use of functional PrnB-GFP chimeric molecules to study the cellular expression and topogenesis of PrnB from its native promoter (Tavoularis *et al.* 2001). Here, this system was used (see Figure 5 and Experimental procedures) to study selected *prnB* mutations, which affect proline uptake kinetics (K245L, K245E, F248L, T456N). Fluorometric quantification in cell-free crude extracts (see Experimental procedures and Tavoularis *et al*. 2001) showed that all mutants expressed 97–120% of the wild-type levels of GFP (results not shown). Figure 5(*a*) shows that all strains analysed carry a single copy of *prnB-gfp* integrated at the resident *prnB* locus, while Figure 5(*b*) shows the cellular expression of PrnB in the wild-type and the mutant strains, as seen by confocal laser microscopy. It is apparent that while mutations K245L and T456N affect partially the localization of PrnB in the plasma membrane, mutations K245E and F248L exhibit physiological cellular expression of PrnB and should, thus, directly affect PrnB function.

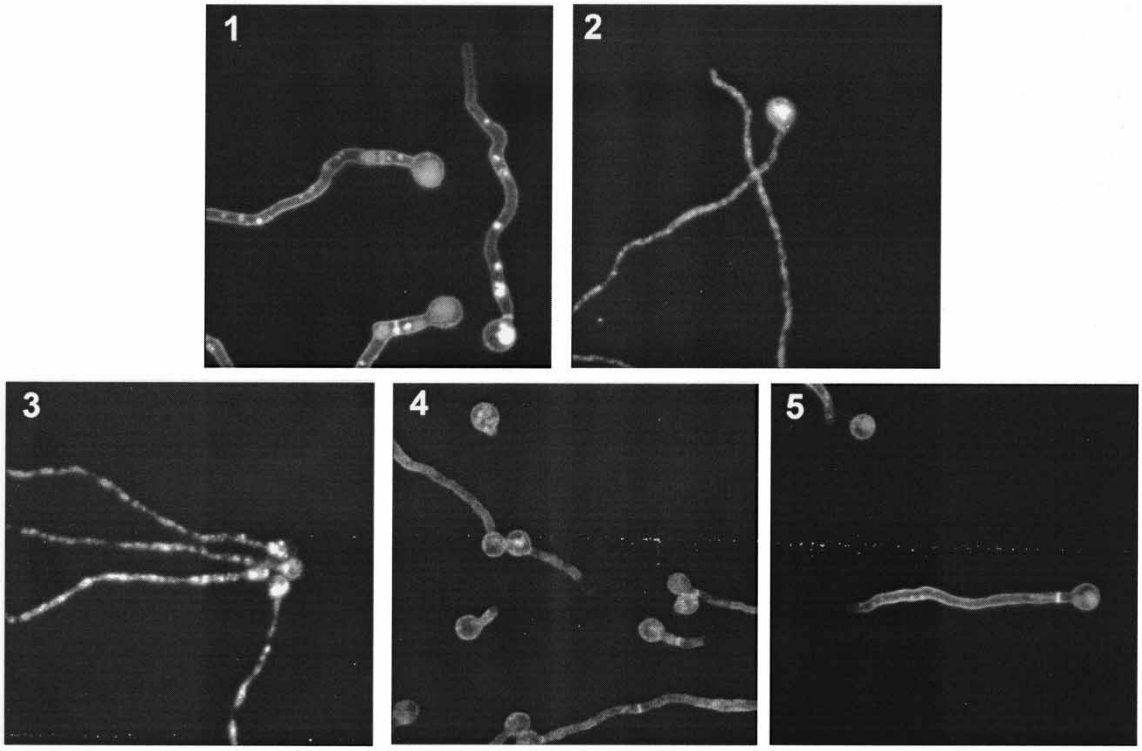
One has also investigated whether the presence of GFP affects the kinetics of different PrnB mutants. Similar to wild-type (Tavoularis *et al*. 2001), the presence of GFP connected with a four amino acid linker to the C-terminus of PrnB does not affect the affinity (K_m) of neither wild-type PrnB nor PrnB mutants for proline (results not shown). On the other hand, and as previously reported for the wild-type protein (Tavoularis *et al*. 2001), GFP has a negative effect on the capacity for proline transport, especially at 37°C. V-values in PrnB-GFP mutants are reduced to 50–75% of the values obtained with wild-type PrnB (results not shown). Thus, although GFP has not been an entirely silent tag, it has proved to be an excellent tool to investigate PrnB cellular expression in wild-type and *prnB* mutants.

Implications of the topology of PrnB functional mutations

Figure 6 shows the predicted topology of PrnB based on computer programmes (PRED-TMR algorithm, see Experimental procedures) utilizing multiply aligned APC transporters and experimental data from biochemical studies with homologous proteins, PheP (Pi and Pittard 1996, Cosgriff *et al*. 2000), AroA (Cosgriff and Pittard 1997, Cosgriff *et al*. 2000), LysP (Ellis *et al*. 1995), GabP (Hu and King 1998a) and Gap1p (Gilstring and Ljungdahl 2000). It must also be stressed that, using the same algorithms, none of the missense or duplication mutations described herein seem to alter significantly the hydrophobicity of any TMS. With the exception of a single mutation (G403A), all missense mutations affecting PrnB function map in the borders of cytoplasmic loops with transmembrane domains and in TMS6. The distribution of PrnB mutations to analogous findings was compared with other APC transporters. As already discussed above, it has been shown that specific mutations in TMS6 of Bap1p and Hip1p in *S. cerevisiae* led to transporters with altered substrate specificity (reduced amino acid uptake and the novel ability to take up K^+ ions). Altered substrate specificity mutations have also been identified in L1 (P113), L2-TMS3 (P148, V149, S152, Y173), L6 (G308, P313), L7 (Y356) and TMS10 (W451, F461) of the arginine transporter of *S. cerevisiae* (Can1p) and in TMS3 of the glutamine/asparagine transporter of *S. cerevisiae* Gnp1p (W239) (Regenberg and Kielland-Brandt 2001). In PheP (*E. coli* phenylalanine transport), it has been shown that mutations affecting function are located in the N-terminal loop (R26, H27), L2-TMS3 (Y92, W95, F101, W105, E118), L4-TMS5 (E159, E161, K168), L6 (E226), TMS7 (R252) and L8 (R317). In addition, insertions of single Ala residues in L8-TMS9 had major effects on PheP transport activity (Pi *et al*. 1998). In AroP (*E. coli* aromatic amino acid transport), site-directed mutagenesis has established that a key residue involved in tryptophan transport is Y103 (Cosgriff *et al*. 2000). In other words, although mutations affecting the function of APC transporters map in various segments of different proteins, at least three regions seem of primary importance: L2-TMS3, TMS6 and L8-TMS9. The importance of L2-TMS3 is strengthened from the observation that, in all relevant studies, mutations affecting the function of amino


prnB397
TpBGFPΔ
TpA4
prnB-T456NGFP
prnB-K245LGFP
prnB-K245EGFP
prnB-F248LGFP
4.15kb
1.80kb

(a)


1
2
3
4
5

(b)

Figure 5

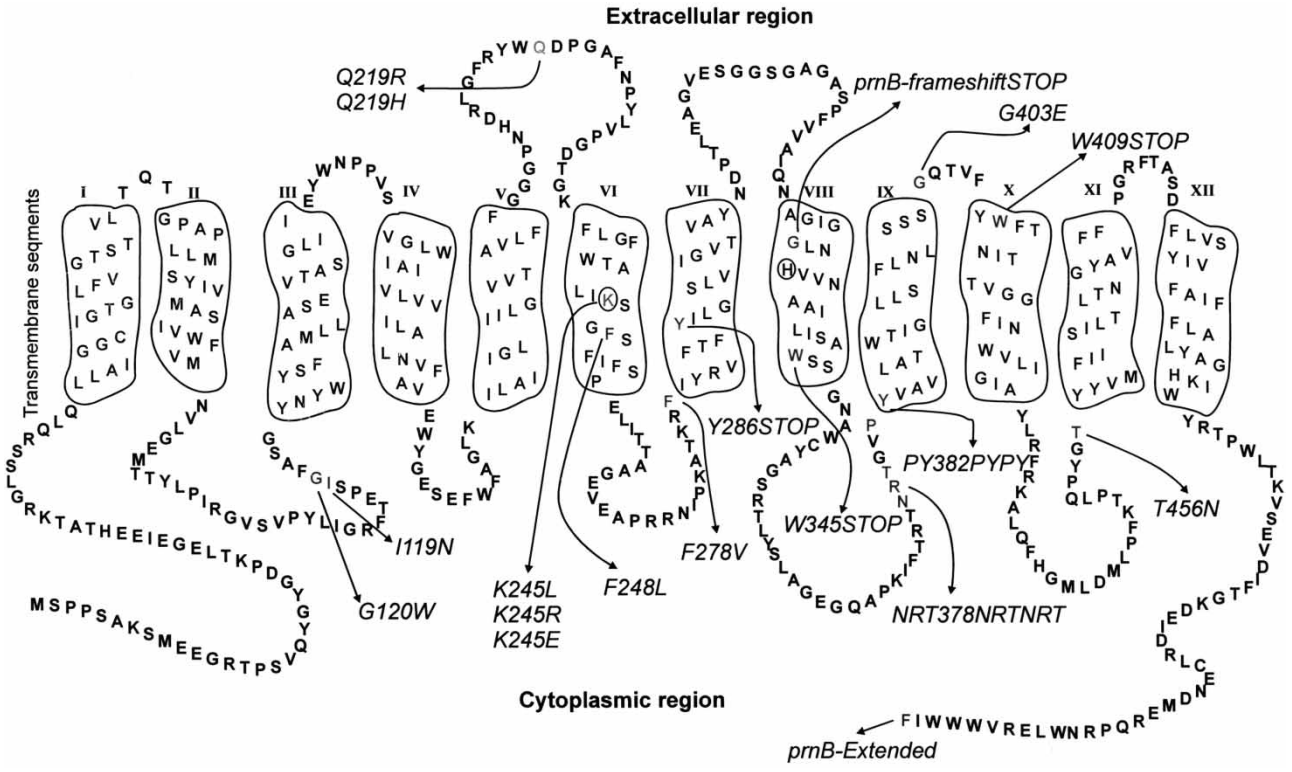


Figure 6. Predicted location of PrnB mutations. The PrnB polypeptide chain is shown crossing the membrane 12 times in a zigzag fashion with the N- and C-termini retained in the cytoplasm. This model has been drawn on the basis of the hydropathy blot and the 'positive-inside' rule (Von Heijne 1992), as described in Experimental procedures and the text. The transmembrane domains shown in boxes contain 19–22 amino acid residues. The two TMS charged residues (K245 and H334) are indicated by circles.

acid transporters were identified within this region. The role of TMS6 in substrate binding and transport is strongly supported by studies involving three different transporters, PrnB (herein), Bap1p and Hip1p (Wright *et al*. 1997). Finally, the importance of L8-TMS9 is underlined by the similar negative effects caused by insertions (or duplications) of amino acids in PheP and PrnB. Interestingly, this region corresponds to a consensus amphipathic region (CAR) shared by many APC transporters and the non-homologous mammalian GABA transporters active in the nervous system (Sophianopoulou and Diallinas 1995, Hu and King 1998b). The CAR has been also shown to be functionally significant in both GabP (Hu and King 1998a) and the mammalian mouse cationic amino acid transporters MCAT (Closs *et al*. 1993). The channel forming nature of the CAR suggests that it might be part of the substrate translocation pathway and that insertions or duplications of amino acid residues might directly affect its function or alter its topology relatively to other domains involved in amino acid binding and transport.

Conclusions

None of the previous studies addressing structure-function relationships in APC transporters has directly shown whether the mutations described have affected the cellular expression and translocation of transporters in the plasma membrane or the actual transport function of the protein. In a single study, a number of non-functional or altered specificity AroP-PheP chimeric proteins, expressed from high copy plasmids, have been studied using immunological detection with PheP-specific antisera (Cosgriff *et al*. 2000). This work studied mutations in an amino acid transporter expressed form its native promoter. It was shown that neither transcription nor translation are affected in different *prnB* mutants, as judged by northern blots and fluroscence quantification analyses of PrnB-GFP chimeras. It was also shown that mutant transporters still depend on a proton gradient across the plasma membrane. Using laser confocal microscopy, it was shown that mutations could be classified to two main groups: Those with partially defective topogenesis in the

Figure 5. Construction and analysis of isogenic strains carrying wild-type and mutant *prnB-gfp* genes. (*a*) Southern blot analysis of total genomic DNA extracted from the recipient strain *prnB397*, the control strains TpBGFPΔ and TpA4 and the mutant strains *prnB-T456NGFP*, *prnB-K245LGFP*, *prnB-K245EGFP* and *prnB-F248LGFP* (for strain details see Experimental procedures). ~10 μg of genomic DNA from each strain were digested with *Pst*I, transferred onto nitrocellulose filters and hybridized with a *prnB-sgfp* specific probe (as described in Experimental procedures and Tavoularis *et al*. 2001). The ~4.15 kb seen in all transformants corresponds to the homologous integration of a single *prnB-gfp* chimeric gene in the *prnB* genomic locus (Tavoularis *et al.* 2001). Strain TpBGFPΔ yields the expected ~1.8 kb band. Molecular weight markers are indicated in kb. (*b*) Representative photos from confocal laser microscopy of TpA4 (1) and the mutant strains, *prnB-T456NGFP* (2), *prnB-K245LGFP* (3), *prnB-K245EGFP* (4) and *prnB-F248LGFP* (5), grown under induced conditions (20 mM proline-glucose) for 16 h at 25°C.

plasma membrane and those with no obvious cellular defect but reduced transport activity. Kinetic analysis of the latter clearly demonstrated that some mutants are directly affected in proline binding and transport (notice the lack of transport activity reflected in the lack of growth of PrnB-F248L on proline, despite an absolutely physiological cellular expression at 25°C). The molecular and genetic tools described herein will allow one to investigate in detail aspects of APC transporters such as interactions with their substrates or interactions with chaperones involved in their controlled subcellular localization.

Experimental procedures

Media, growth conditions and strains for A. nidulans

Minimal (MM) and Complete (CM) media and growth conditions for *A. nidulans* have previously been described (Cove 1966). Supplements were added when necessary. Nitrogen sources, urea and proline were used at a final concentration of 5–10 mM. Uric acid was used at a concentration of 595 μM. The carbon sources, glucose and ethanol, were used at final concentrations of 1% w/v and v/v, respectively. The *A. nidulans* strains used have the following genotypes: *yA*$^+$ *pabaA1* (*prnB*$^+$), *yA2 pantoB100* (*prnB*$^+$), *argB2 biA1 pantoB100*, *yA2 pabaA1 prnB377* (*prnB377*), *yA2 pabaA1 riboB2 pantoB100 prn397* (*prnB397*) and *yA2 pabaA1 sasA60*. *prn397* is a deletion starting at the *Pst*I site of the *prnB* gene and extending up to the *Pst*I site of the *prnC* gene (Tavoularis *et al*. 2001). *prnB377* is a deletion in the open reading frame of the *prnB* gene that was described previously in Tazebay *et al*. (1995). TpBGFPΔ and TpA4 strains were derived from *prn397* strain transformed with plasmids pBC9 and pA4, respectively (Tavoularis *et al*. 2001). *prnB-T456NGFP*, *prnB-K245LGFP*, *prnB-K245EGFP* and *prnB-F248LGFP* strains were derived from *prn397* transformed with plasmids pA4 carrying the corresponding mutant *prnB* alleles constructed by *in vitro* site-directed mutagenesis (Ex-Site PCR-Based Site-Directed Mutagenesis Kit; Stratagene, La Jolla, CA). In all cases, the sequence of the entire *prnB and gfp* open reading frames was determined using the ABI 310 Genetic Analyser at the Institute of Biology, NCSRD, Athens, Greece. All other strains including *prnB* alleles isolated in this work were either selected from *yA2 pabaA1 sasA60* strain or constructed in strain *yA2 pabaA1 riboB2 pantoB100 prn397*. *pantoB100*, *pabaA1*, *riboB2*, *biA1* and *argB2* indicate auxotrophies for D-pantothenic acid, *p*-aminobenzoic acid, riboflavine, biotin and arginine, respectively. *yA*$^+$ indicates green conidia, while *yA2* results in yellow conidia. These markers do not affect the regulation of gene products involved in proline uptake and catabolism. The *sasA60* mutation leads to toxicity of compounds, which can be converted to semi-aldehydes such as L-proline and GABA (γ-amino-n-butyrate). The toxicity of L-proline to *sasA60* strains is such that mutations conferring resistance can be selected (Arst *et al*. 1981).

DNA manipulations and protoplast transformation

Plasmid isolation from *Escherichia coli* strains and standard DNA manipulations were performed as previously described (Sambrook *et al*. 1989). Polymerase Chain Reaction (PCR) was carried out using AmpliTaq DNA polymerase (Perkin-Elmer) and the Expand High Fidelity PCR system (Roche Molecular Biochemicals, Mannheim, Germany). DNA sequencing of plasmid constructions and PCR products were carried out using the ABI 310 Genetic Analyser at the Institute of Biology (NCSRD, Athens, Greece). The oligonucleotide primers, specific for the *prnB* gene, used for sequencing and PCR amplifications were:

PA: 5′CTGGGGAATTCCCGCTCAGGAATCACTT3′
BA: 5′CCAGCCGGGACTC TTCTC3′
UA: 5′CCCCCGTCGGCCAAGAGC3′
P6: 5′GTCCTGGGCGAGATGACC3′
P1: 5′GCCCTGAACGTCTTCGCG3′
P9: 5′GGTTCCCGGGGACACTGG3′
P3: 5′GGAACATCCCCAAAGCC3′
P7: 5′CGCTCGCCGGTGAGGG3′
P109: 5′AACGGGTACGCGGTGTTCTT3′
P117: 5′GATCTCTGCCTGGTCATC3′
BB: 5′GCGATAACTATCTGGTAG3′
UB: 5′CCACCACCAGACTCGCTCC3′
P8: 5′CCGAAGCAGTGAAGCGGC3′
P4: 5′AGAACAGCCACATAGGG3′
P11: 5′CCAGCCTCAAGGGTAGGG3′
P2: 5′AGAAGGTGAAAACACGG3′
P5: 5′CTCCGTACCACTCGACC3′
P10: 5′CGGTGAAGCGGCCGATC3′

The oligonucleotide primers used for the *in vitro* construction of *prnB* alleles were:

Q219E: 5′CCGCTACTGGGAAGACCCGGTGC3′
Q219R: 5′CCGCTACTGGCGAGACCCCGGTGC3′
Q219H: 5′CCGCTACTGGCACGACCCCGGTGC3′
K245R: 5′GCCCTGATCAGGTCCGGTTTTTCG3′
K245E: 5′GCCCTGATCGAGTCCGGTTTTTCG3′
K245L: 5′GACTGCCCTGATCCTGT CCGGTTTTTCG3′

A. nidulans protoplast transformation was carried out as described by Tilburn *et al*. (1983). Total genomic DNA isolation from *A. nidulans* strains and Southern blot analysis has been carried out as in Lockington *et al.* (1985). The DNA fragment used as a probe in Southern blots was a ~2 kb *PpuMI-BamHI* restriction fragment of plasmid pA4 (Tavoularis *et al.* 2001). Northern blot analysis has been carried out using the glyoxal method described by Tazebay *et al*. (1997). The DNA fragment used as a probe in northern blots was a ~1.8 kb *Pst*I restriction fragment of the *prnB* gene (Sophianopoulou and Scazzocchio 1989) isolated from plasmid pAN225 (Hull *et al*. 1989, Tavoularis *et al.* 2001). The *A. nidulans* actin gene (*acnA*) is used as an internal control to monitor the amount of RNA loaded in different lanes (Fidel *et al.* 1988, Tazebay *et al*. 1997).

Genetic isolation of prnB mutations

Early genetic work had led to the isolation of several *prnB*-specific mutations (*prnB6*, *prnB50*, *prnB32*, *prnB36*, *prnB81*, *prnB82*, *prnB109*, *prnB1110*, *prnB206*) (Arst and MacDonald 1975, Arst *et al*. 1981, Jones *et al*. 1981, Arst Jr., H. N., Personal communication). Those used in the present work were selected either as spontaneous mutations conferring resistance to 50 mM L-proline in a strain of genotype *proA6 sasA60* (*prnB81*, *prnB82*) or *proB9 prnD156* (*prnB206*), as described by Arst *et al.* (1981), or after treatment of a strain of genotype *proA6* with N-methyl-N′-nitro-N-nitrosoguanidine (NTG) (*prnB6*, *prnB32* and *prnB36*), as described by Arst and MacDonald (1975). The toxicity of L-proline to *sasA60* strains is such that mutations conferring resistance can be selected (see above, Arst *et al*. 1981). The *proA6* mutation results in an L-proline requirement, which facilitates screening of *prnB* mutations, distinguishing them from mutations in other genes (Arst *et al*. 1980, Tazebay *et al*. 1995). This work modified the original genetic selection in order to isolate partial loss-of-function or conditional *prnB* mutations. *prnB* mutants were selected on an *A. nidulans* strain with a *sasA60* genetic background (*yA2 pabaA1 sasA60*), as spontaneous or UV-induced sectors conferring resistance to 50 mM L-proline, as described by Arst *et al*. (1981), at both 25 and 37°C. All mutant strains were then tested at both temperatures, in order to recognize cryo- and temperature sensitive mutants. Selected putative *prnB* mutants were crossed to a strain *argB2 biA1 pantoB100* to segregate out the *sasA60* mutation.

Construction of targeted prnB alleles and prnB-gfp chimeric genes

In vitro prnB directed mutagenesis was performed by the method of Kunkel *et al.* (1987) using plasmid pBHX1. This plasmid contains the *Eco*RI-*Hind*III fragment of plasmid pAN225 including the *prnB* gene (Tavoularis *et al*. 2001). The codon CAA^{758} that specifies amino acid residue Q219 of PrnB was substituted for CAC^{758} (mutation H219) and CGA^{758} (mutation R219). The codon AAG^{836} that specifies amino acid residue K245 of PrnB was substituted for GAG^{836} (E245), CTG^{836} (L245) and AGG^{836} (R245). The fragments *Ppu*MI-*Sph*I ($\sim$1.2 kb) of the plasmid pBHX1 containing the mutations were sequenced to verify that none contains any other mutations and were used to substitute for the same fragment of plasmids pBC9 or pA4. Plasmid pBC9 contains a $\sim$4.9 kb *EcoR*I-*Pfl*MI fragment, derived from plasmid pAN225, containing the *prnB* gene and part of the *prnC* gene in a bluescript KS(+) vector (Tavoularis *et al.* 2001). pA4 is a version of pBC9 in which the *prnB* open reading frame is fused in-frame with the *gfp* open reading frame via a specific four amino acid linker (Tavoularis *et al.* 2001). Upon transformation of the mutant strain *prn397*, which carries a large deletion extending from within the *prnB* open reading frame to within the *prnC* open reading frame, with linearized pBC9 or pA4 plasmids containing the mutations, transformants are directly isolated on proline as the sole nitrogen source. Strain *prn397* lacks both PrnB and PrnC (L-Δ^1-pyrroline-5-carboxylate dehydrogenase) activities and does not grow at all on media containing proline as a sole nitrogen source, as the absence of PrnC results in strong proline toxicity. This strain allows the direct selection of $prnC^+$ transformants upon reintroduction of sequences containing any *prnB* allele and the missing *prnC* sequences, notwithstanding whether or not the introduced *prnB* sequences are functional. This is because even complete loss-of-function mutant in *prnB* (see below) allows leaky growth on proline, which can easily be assessed on the background of the *prnB-prnC* deleted strain. The functionality of the PrnB protein can then be assessed directly by growth on proline as a sole nitrogen source (Tavoularis *et al*. 2001). This system leads to the targeted, single-copy integration of any *prnB* allele or *prnB-gfp* chimeric gene at the resident *prnB* locus, thus avoiding complications arising from ectopic and/or multiple integrations of *prnB* copies.

Confocal laser microscopy

Samples were prepared as described previously in Tavoularis *et al.* (2001). Confocal laser microscopy was carried out on a BIO-RAD MRC 1024 CONFOCAL SYSTEM, Laser Sharp Version 3.2 Bio-Rad Software, Zoom $\times$4, Excitation/emission: 488nm/Blue, Samples at Laser Power 10%, Kalman filter $n = 5$–6, 0.3 μm cut, Iris: 7–8, Crypton/Argon Laser. Nikon DIAPHOT 300 Microscope, $\times$60 (Oil immersion) Lens Emission Filter 522/DF35. Lens Reference: Plan Apo 60/1.40 oil DM (Nikon, Japan) 160175, 60 DM/Ph4, 160/0.17.

Fluorometry

Samples were prepared as described previously in Tavoularis *et al.* (2001). A Perkin Elmer Fluorescence Spectrophotometer MPF-3 was used. Emission was detected at 510 nm, $\Delta\lambda = 6$ nm when 488 nm, $\Delta\lambda = 4$ nm was used as the excitation wavelength. Values were normalized against the amount of total protein in the samples and expressed as relative GFP fluorescence units per micrograms of protein. Protein concentration was determined using a modified Bradford assay (Bradford 1976).

Proline transport assays

[2,3,4,5-^{3}H] L-proline uptake was assayed in germinating conidia at 37°C, as previously described (Tazebay *et al*. 1995, Meintanis *et al*. 2000). Standard uptake assays for the determination of initial uptake rates were performed in *A. nidulans* MM (pH 6.5) by using 10 μM [2,3,4,5-^{3}H] L-proline (specific activity 120 Ci mmol^{-1}; Moravek, Biochemicals, Brea CA, USA). Initial uptake rates were expressed in pmol of substrate incorporated per 1 min per 10^8 viable conidia. Radioactivity was determined in sediment and supernatant by liquid scintillation counting (Beckman Instruments). Transport measurements were repeated independently and the reported results represent the mean values of at least three-to-five different experiments. The apparent Michaelis constant (K_m) and maximal velocity (V) values for ^{3}H L-proline were determined from double reciprocal plots of the initial uptake rates against substrate concentration. Initial uptake rates were corrected by subtracting background uptake values, evident in the *prn377* total loss-of-function mutant strain, calculated in every uptake experiment (Tazebay *et al*. 1995). The errors given are standard deviations of the mean value.

In silico *analyses*

Sequences were collected from the SwissProt database using the programme BLASTP (Altschul *et al*. 1997) and were aligned using the programme CLUSTAL-X (Thompson *et al*. 1997). Predicted topology of PrnB based on computer programmes (PRED-TMR algorithm; http://biophysics.biol.uoa.gr/PRED-TMR; Pasquier *et al*. 1999) and experimental data from biochemical studies with homologous proteins.

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