FEBS Letters 579 (2005) 3984–3990 FEBS 29737

The coupling mechanism of *P*-glycoprotein involves residue L339 in the sixth membrane spanning segment

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Received 17 May 2005; revised 6 June 2005; accepted 9 June 2005

Available online 28 June 2005

Edited by Sandro Sonnino

Abstract The transmembrane (TM) domains in *P*-glycoprotein (*P*-gp) contain the drug binding sites and undergo conformational changes driven by nucleotide catalysis to effect translocation. However, our understanding of exactly which regions are involved in such events remains unclear. A site-directed labelling approach was used to attach thiol-reactive probes to cysteines introduced into transmembrane segment 6 (TM6) in order to perturb function and infer involvement of specific residues in drug binding and/or interdomain communication. Covalent attachment of coumarin-maleimide at residue 339C within TM6 resulted in impaired ATP hydrolysis by *P*-gp. The nature of the effect was to reduce the characteristic modulation of basal activity caused by transported substrates, modulators and the potent inhibitor XR9576. Photoaffinity labelling of *P*-gp with [^{3}H]-azidopine indicated that residue 339C does not alter drug binding per se. However, covalent modification of this residue appears to prevent conformational changes that lead to drug stimulation of ATP hydrolysis.
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Keywords: *P*-glycoprotein; Multidrug resistance; Coupling; Drug transport

1. Introduction

The "*multidrug*" transporters such as *P*-glycoprotein (*P*-gp or ABCB1) are at the heart of many serious clinical problems including resistance to antibiotic and cancer chemotherapy. Due to its clinical importance in cancer therapy, *P*-gp remains one of the most investigated "*multidrug*" transporters, however, many fundamental aspects of its transport mechanism have not been established. For example, a largely unexplained characteristic of *P*-gp is the ability to interact with an extraordinarily broad range of chemically and functionally unrelated compounds. This characteristic of *P*-gp arises through the presence of multiple, allosterically linked, but pharmacologically distinct drug binding sites on the protein [1–5]. Early photoaffinity studies suggested that the binding sites comprise elements from both halves of the protein [6,7]. Evidence obtained from mutational studies [8,9], mass spectroscopy data [10] and cysteine-scanning mutagenesis [11,12] indicates that several TM segments are involved in the drug binding site, with TMs 5, 6, 11 and 12 featuring prominently.

Transport from these drug binding sites is mediated in an ATP-dependent fashion and a widely accepted model suggests that the two nucleotide binding domains (NBDs) hydrolyse ATP in an alternating and co-operative manner [13]. In addition, the NBDs and drug binding sites display co-operativity during *P*-gp function as evidenced by the ability of drugs to stimulate nucleotide hydrolysis. For a coupled active transport mechanism there needs to be communication in both directions (i.e. NBD → TMD and TMD → NBD) to effect vectorial drug movement [14]. Unfortunately, there is a paucity of information on the molecular regions involved in mediating communication in either direction.

That there is two-way coupling with concomitant conformational changes has been established by numerous investigations, including tryptic digestion [15,16], binding of the conformationally sensitive antibody UIC2 [17], and spectroscopic studies involving trypotophan fluorescence quenching or ^{2}H/H-exchange profiles [18,19]. Investigations using electron microscopy of 2D crystals have confirmed that functionally relevant structural changes accompany progression through the catalytic cycle [20,21]. Unfortunately, none of these techniques are at a sufficient resolution to identify specific regions of *P*-gp, or individual amino acids, that are involved in mediating the conformational changes.

Recently we provided evidence that transmembrane segment 6 (TM6) undergoes distinct topographical changes in response to stimuli emanating from the NBDs [22]. This approach, using purified and reconstituted protein, allowed direct assignment of functional alterations to *P*-gp, in contrast to studies of TM6 topography in membranes containing *P*-gp [20]. TM6 is of interest since it is directly linked to the N-terminal NBD in *P*-gp and therefore a likely candidate for communication pathways. Furthermore, several mutations in TM6 caused by drug selection altered the resistance profile [23], as did directed mutagenic investigations [24]. In the current investigation site-directed cysteine labelling was used to provide information

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Abbreviations: *P*-gp, *P*-glycoprotein; MDR, multidrug resistance; ABC, ATP Binding Cassette family; TM6, transmembrane segment 6; NBD, nucleotide binding domain; ANOVA, one-way analysis of variance; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; S.E.M., standard error of the mean

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doi:10.1016/j.febslet.2005.06.030

on the functional role of TM6 in drug translocation. Single cysteine containing *P*-gp isoforms were labelled covalently with coumarin-maleimide (CM) and the effect of this modification on function assessed. The results identify residue 339 in *P*-gp as being involved in conformational changes required to communicate drug binding events to the NBDs.

2. Materials and methods

2.1. Materials

Coumarin-maleimide was purchased from Molecular Probes (Leiden, NL). Octyl-β-D-glucoside, paclitaxel and Ni–NTA His Bind Superflow resin were obtained from Merck Biosciences (Nottingham, UK). [^{3}H]-Azidopine (59 Ci/mmol) was purchased from Amersham Biosciences (Chalfont St. Giles, UK). Crude *Escherichia coli* lipid extract was obtained from Avanti Polar Lipids (Alabaster, USA). Insect-Xpress medium was purchased from Cambrex BioScience (Nottingham, UK) and Excell 405 from AMS Biotechnology (Abingdon, UK). XR9576 was a kind donation from Xenova Ltd. (Slough, UK). All other reagents were of at least analytical grade and were from Sigma (Poole, UK).

2.2. Production of recombinant baculovirus

Expression and characterisation of a cysteineless, histidine-tagged *P*-gp in mammalian cells has been described [25,26]. Single cysteines were introduced into the cysteineless multidrug resistance 1 coding sequence by site-directed mutagenesis, employing the mutagenic oligonucleotides (5′–3′; in each case the cysteine codon is in bold):

oligoV331C, TCTATTGGACAA**TGC**CTCACTGTA; oligoT333C, ATTGGACAAGT*T*CTC**TGC**GTATTCTTTTC; oligoF335C, CTCACTGTA**TGC**TTTTCTGT*G*TTAATTGGG; oligoS337C, CTGTATTCTTT**TGT**GT*G*TTAATTGGG; oligoL339C, CTTTTCTGTA**TGC**ATTGGGGCT; oligoG341C, CTGTATTAATT**TGC**GC*A*TTTAGTGTTGG; oligoF343C, TTTTCTGTATT*G*ATTGGGGCT**TGT**AGTGTTGG.

The nucleotide sequence of the mutated DNA fragments were verified by DNA sequencing. The entire coding sequence for cysteineless-*P*-gp and single cysteine mutants, including the 5′ Kozak sequence, was introduced into the baculoviral transfer vector pBacPAK9 (Clontech) and recombinant baculoviruses generated by in vivo recombination with BacPAK6 (Clontech).

2.3. Expression, purification and reconstitution of P-gp from insect cell membranes

Trichoplusia ni (high-five) cells were infected with recombinant baculovirus and *P*-gp containing membranes isolated as previously reported [22,26]. Membranes were resuspended in buffer (10 mM Tris–HCl, 150 mM NaCl, 1.5 mM $MgCl_2$, 20% glycerol, 0.4% (w/v) lipids, 20 μM leupeptin, 1 mM benzamidine, 1 μM pepstatin, and 2% (w/v) octyl-β-D-glucoside, pH 7.4) to solubilise protein [22,26]. Soluble proteins were added to Ni–NTA resin at a ratio of 0.5 ml resin to 50 mg soluble protein, washed, and eluted using a stepwise gradient of imidazole. Fractions containing *P*-gp were reconstituted by SM-2 BioBead mediated detergent adsorption and the efficiency of reconstitution assessed by examination of protein and lipid migration through sucrose density gradients as described [26]. Protein concentration was determined by densitometric analysis of Coomassie-stained sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) gels [22].

2.4. Measurement of ATP hydrolysis

ATP hydrolysis was determined using minor modifications [26] of a colorimetric procedure [27] to measure the liberation of inorganic phosphate (P_i). Proteoliposomes (~0.3 μg *P*-gp) were incubated in the presence of varying ATP concentrations (0–1.75 mM) for 20 min at 37 °C to determine the "basal" rate of hydrolysis. To determine the stimulatory effects of drugs, the assays were also done in the presence of 30 μM of the transported substrate vinblastine or the modulator nicardipine. The ATPase activity was plotted as a function of ATP concentration and the relationship fitted, using non-linear regression, with the Michaelis–Menten equation. Alternatively, the activity was measured at constant ATP (2 mM) and varying drug concentration (10^{-9}–10^{-4} M). The relationship between activity and drug concentration was fitted with the general dose–response equation [28].

2.5. Labelling of cysteine containing P-gp isoforms with CM

Samples of each purified, reconstituted *P*-gp isoform (1.5–3 μg) were incubated with CM (10 μM) for various times between 0 and 300 min at 20 °C in the dark. Coumarin-maleimide was added from concentrated DMSO based stocks with solvent levels not exceeding 0.5% (v/v). The reaction was terminated with dithiothreitol (100 μM) and the samples placed on ice. Each sample was then diluted 1:1 with glycerol free buffer (150 mM NaCl, 1.5 mM $MgSO_4$, 0.02% (w/v) NaN_3, and 20 mM Tris–HCl, pH 7.4) and subjected to ultracentrifugation (15 min, 4 °C, 125000 × *g*) to remove unbound probe. Pellets were resuspended in Laemmli sample buffer and proteins resolved using 7.5% SDS–PAGE. A further sample was incubated in 2% (w/v) SDS to denature protein prior to incubation with CM as described above. This sample was used to determine 100% labelling with fluorophore. Gels were analysed using the BioDocIt Imaging system (light source; λ = 302 nm) to determine labelling intensity. The latter was quantified using densitometric analysis (NIH Image Software). Time courses for labelling were plotted as the percent labelling (relative to denatured sample) as a function of time and the exponential association curve was fitted using non-linear regression:

$$L = L_{max} \cdot (1 - e^{-kt}), \quad (1)$$

where L is the percent labelled, L_{max} the maximum percent labelled, t the time (min) and k is the observed rate constant for labelling ($t_{1/2} = \ln 2/k$).

Following imaging, the gels were stained with Coomassie blue to ensure equivalent protein loading.

In order to examine the functional consequences of labelling, samples were treated in a similar fashion to above. The values for half-life of labelling obtained were used in to ensure >90% labelling in each preparation. The final ultracentrifugation step to remove unbound probe was not employed and the samples analysed, immediately following the labelling reaction, for ATPase activity or drug binding.

2.6. [^{3}H]-Azidopine labelling of P-glycoprotein

Preparations of both cys-less and L339C isoforms of *P*-gp were labelled to an extent of 90% with CM as described [22]. Proteoliposomes (0.3 μg) were then incubated in the presence of 0.6 μM [^{3}H]-azidopine in a total volume of 40 μl for 2 h at 20 °C in the dark. Samples were also incubated with radiolabel and an excess (100 μM) of one of the following: vinblastine, paclitaxel, nicardipine or XR9576. Photoactivated cross-linking of [^{3}H]-azidopine to *P*-gp was then achieved by irradiation with UV light for 5 min on ice (100 W, 5 cm). Laemmli sample buffer was added to the samples and proteins resolved using 7.5% SDS–PAGE. Gels were fixed in 25% (v/v) isopropanol, 10% (v/v) acetic acid and washed in Amplify™ prior to autoradiography. The band intensities were quantified using densitometric analysis (NIH Image Software).

2.7. Data analysis

All non-linear regression analyses were produced using the GraphPad Prism 3.0 program. A minimum data set was obtained from at least three independent protein purification preparations and all values are given as means ± standard error of the mean (S.E.M). Multiple data comparisons (>3 sets) were analysed using one-way analysis of variance (ANOVA), with a Newman–Keul post hoc test and statistical significance was presumed, where $P < 0.05$.

3. Results

3.1. Labelling of P-gp isoforms containing cysteine residues within TM6

Single sites for mutation to cysteine within TM6 were chosen based upon structural and topological considerations. Seven individual amino acid substitutions at alternate positions within TM6 were used in order to produce 360° coverage of the α-helical face, and span the hydrophobic core of the lipid

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