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Phosphatidylglycerol dependent protein translocation across the *Escherichia coli* inner membrane is inhibited by the anti-cancer drug doxorubicin

Evidence for an electrostatic interaction between the signal sequence and phosphatidylglycerol

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OmpF-Lpp, a model secretory protein, requires both a positively charged signal sequence and phosphatidylglycerol (PG) for efficient translocation across the *E. coli* inner membrane. Modification of the signal sequence can, however, remove both these prerequisites for translocation providing OmpF-Lpp mutants which undergo either PG and charge dependent or PG and charge independent translocation. Here we show that positively charged membrane interactive compounds (polylysine & doxorubicin) are able to inhibit PG dependent translocation of the OmpF-Lpp signal sequence mutants but not PG independent translocation. Doxorubicin is also shown to bind more efficiently to liposomes containing increased levels of anionic lipid indicating that in these assays it may be inhibiting translocation by preventing electrostatic interaction between the anionic lipid head group and the positively charged signal sequences.

Protein translocation; Phosphatidylglycerol; Signal sequence

1. INTRODUCTION

In prokaryotic systems proteins which are not destined to remain at their site of synthesis are synthesised as precursors with a cleavable N-terminal extension or signal sequence [1]. The signal sequence has a tripartite morphology which consists of a basic N-terminus, a central hydrophobic core and a polar C-terminal region [2]. Within *E. coli* efficient translocation of the precursor requires a number of proteins [3]. One such protein is SecA which is found in a cytoplasmic form and bound to the cytoplasmic face of the inner membrane. SecA may play a role in targeting the precursor to the site of translocation and also acts as an ATPase during the translocation process [4]. The ATPase activity is dependent on the presence of the precursor, anionic lipid and the integral membrane proteins SecE/Y. In addition evidence has been presented which indicates that the translocation process itself is dependent on negatively charged lipid [5,6] but the reason for this dependency is unknown.

We have recently investigated the requirement of translocation for anionic lipid [7] by using two series of OmpF-Lpp signal sequence mutants [8]. Both series contained variable numbers of positively charged lysine residues (0K, 2K or 4K) at the N-terminal end of the signal sequence but in one series the central hydrophobic core region was composed of 8 Leu residues and in the other the core contained 9 Leu residues. This gave rise to two series of mutants 0K8L, 2K8L, 4K8L and 0K9L, 2K9L, 4K9L. It has been shown that the 8 Leu series requires the positively charged N-terminus to be present for efficient translocation and the translocation efficiency increases with increasing positive charge [8]. In addition we recently showed that the translocation efficiency of this series is strongly dependent on the presence of the anionic lipid phosphatidylglycerol (PG) and the PG requirement mimics the charge dependency of the translocation pathway [7]. On increasing the length of the hydrophobic core by incorporating a 9th Leu residue both the charge dependency and PG de-

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pendency of the translocation process are lost. We postulated that the PG dependent translocation path involves an electrostatic interaction between the positively charged signal sequence and the anionic lipid headgroup [7]. In the case of the 9 Leu series the increase in the level of hydrophobicity/length of the core region is postulated to allow the signal sequence to directly interact with the membrane or a component of the translocation machinery and does not require the level of charge stabilisation seen in the case of the less hydrophobic 8 Leu series.

Positively charged compounds which have the potential to interact with anionic lipid headgroups have previously been shown to perturb the efficiency of the translocation reaction [9]. Doxorubicin is an anthracyclin glycoside antibiotic which preferentially binds to anionic lipid at the membrane interface [10,11] thereby impairing processes which are dependent on these lipids [13,14]. We have used both this drug and polylysine to probe the nature of the PG dependency of translocation.

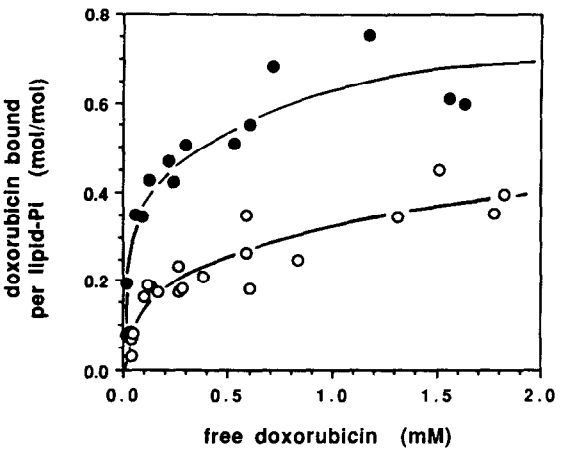

Fig. 1. Doxorubicin binding assay. *E coli* HDL11 was grown in the presence of 0 μM or 60 μM IPTG to produce vesicles containing approximately 5% (○) or 28% (●) of the total lipid as PG and CL. 400 nm LUVETS were prepared from total lipid extracts and incubated with varying concentrations of doxorubicin. The level of doxorubicin bound is recorded per lipid phosphate in the sample. All points are mean values of experiments performed in duplicate.

2. MATERIALS AND METHODS

2.1. *Bacterial strains and plasmids*

E. coli strain MRE600 [15] was used for the isolation of a S-135 extract [16] and in addition this strain was used for the preparation of inverted inner membrane vesicles with a wild type phospholipid composition (19.8% of the total lipid was PG) [5]. Inverted inner membrane vesicles with differing levels of acidic phospholipid were isolated from the strain HDL11 grown in the presence of 0 μM isopropylthiogalactoside (IPTG) (3.5% of total inner membrane lipid was PG) or 60 μM IPTG (19.1% PG) as previously described [6].

Plasmids 2K8L, 4K8L, 2K9L, 4K9L were used for the in vitro expression of the OmpF-Lpp mutants 2K8L, 4K8L, 2K9L, 4K9L respectively [8].

2.2 *Materials*

SecA [17], and SecB [18] were purified as described, [^{35}S]methionine (1000 Ci/mmol) was obtained from Amersham, IPTG was obtained from Sigma.

2.3. *In vitro transcription-translation and translocation reactions*

The in vitro reactions were performed as previously described [5] but SP6 polymerase (Pharmacia) was used in the transcription of the plasmids at 37°C for 45 min. The phospholipid content based on Pi was determined after phospholipid extraction, [19], using thin layer chromatography and perchloric acid destruction [20] The chromatography plates were impregnated with 1.2% boric acid in ethanol (1.1) and dried before use. The solvent system used was chloroform/methanol/water/ammonium (25%) (65:37:5:3:1) v/v [21]

After transcription translation was allowed to continue for ten minutes, the mixture was diluted 1:1 with 12 M urea in 50 mM sodium phosphate buffer pH 7.4, followed by 3 min sonication in a water bath to prevent aggregation of the precursor proteins [8].

The translocation reactions were performed at 37°C as previously described [5] but where necessary, prior to the assay, the membrane vesicles were incubated for 2 min at 37°C in the translocation assay mixture containing the positively charged compounds (doxorubicin or polylysine). The reaction was only allowed to proceed for 5 min after which proteinase K was added to digest all of the non-translocated protein. After incubating for a further 10 min at 37°C the protease treatment was stopped by the addition of trichloroacetic acid to a final concentration of 15%. After precipitating on ice the samples were analysed by tricine SDS-PAGE [22] and fluorography.

In vitro translocation is defined as the amount of precursor and mature form of the protein remaining after proteinase K treatment. The level of translocation was quantified by scintillation counting of rehydrated, excised protein bands (precursor and mature) from the dried gels

To calculate translocation efficiency the percentage of the available precursor which had translocated across wild type vesicles prepared from MRE600 was normalized to 100%. The level of translocation observed after the vesicles had been pre-incubated with either polylysine or doxorubicin was related to this 100% level

2.4. *Binding experiments*

400 nm, large unilamellar vesicles prepared by extrusion (LUVETS) (800–1000 nmol phosphate) [11], were mixed with variable concentrations of doxorubicin in a final volume of 1 ml The buffer used was 10 mM PIPES, 100 mM NaCl, 0.5 mM EGTA (triplex VI), pH 7.4 (NaOH). The assay mixture was incubated for 1 and 3 h in the dark (both time intervals gave identical results) at room temperature (20–25°C) The LUVETS were pelleted (60 min, 435,000 × *g*, Beckman TLA100.3) A sample of the supernatant was diluted for determination of the equilibrium drug concentration Binding of drug to the tubes and the amount of lipid in the supernatant were both negligible.

3. RESULTS AND DISCUSSION

Translocation of the OmpF-Lpp signal sequence mutants containing a hydrophobic core of 8Leu residues is dependent on anionic phospholipid [7] and it has been postulated that the anionic lipid is electrostatically interacting with the signal sequence at some stage within the translocation pathway. Since doxorubicin is also known to interact with membranes containing anionic lipid [11,12] we investigated whether doxorubicin binding was able to inhibit the translocation process.

Initially it was necessary to show that there was a

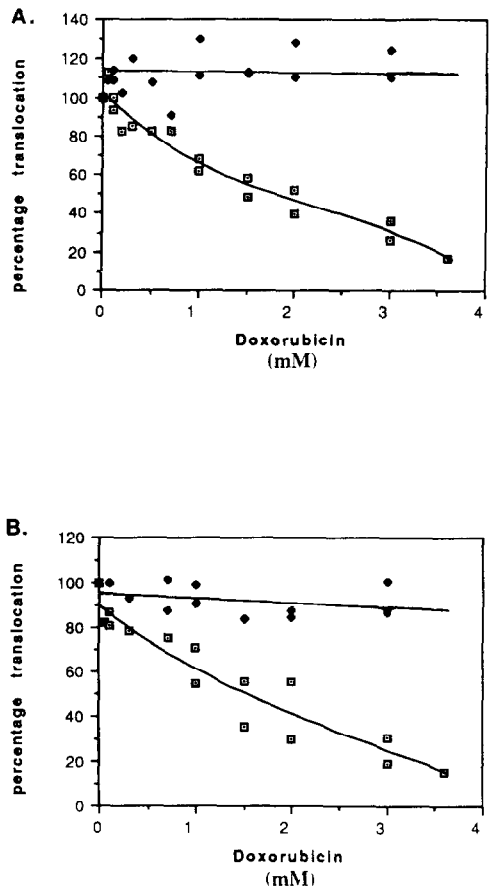

Fig. 2. Effect of doxorubicin on translocation. Translocation assays were performed using wild type membrane vesicles (MRE600). The effect of doxorubicin on the translocation efficiency of the 2K8L (□) and 2K9L (◆) signal sequence mutants are shown in (A). (B) shows translocation efficiency of 4K8L (□) and 4K9L (◆) in the presence of varying concentrations of doxorubicin. 100% represents wild type translocation. Samples were analysed by tricine SDS-PAGE and fluorography and quantified by scintillation counting. All points are the mean of two to three experiments

relationship between the doxorubicin interaction at the membrane interface and the level of anionic lipid in the bilayer. HDL11 is an *E. coli* strain in which the *pgs*A gene encoding phosphatidylglycerol phosphate synthase has been placed under the control of a lac promoter. By incubating the bacteria in the presence of varying levels of IPTG it is therefore possible to control the level of *pgs*A expression and hence control the level of PG and cardiolipin (CL) production [6]. In the absence of IPTG the level of anionic lipids within the inner membrane was found to be 2% PG and 3% CL based on the total phospholipid but incubation in the presence of 60 μM IPTG produced cells containing 28% of the total lipid as PG and CL. Total lipid extracts from cells grown in the presence and absence of IPTG were used to produce 400 nm LUVETS [11] which were incubated in the presence of varying doxorubicin concentrations. As can be see in Fig. 1 increased levels of anionic lipids within the membrane increases the level of doxorubicin binding to the LUVETS implying that the doxorubicin is interacting preferentially with the anionic lipids, presumably via an electrostatic interaction. We then proceeded to investigate the effect of doxorubicin on translocation of the OmpF-Lpp mutants. Fig. 2 shows that in the case of the OmpF-Lpp mutants which require PG for translocation, increasing the level of doxorubicin inhibits the level of translocation across *E. coli* inner membranes with a wild type lipid composition. Importantly the PG independent translocation of the 9 Leu OmpF-Lpp mutants was unaffected by the level of doxorubicin present and the mutants were able to translocate at wild type levels in all cases.

It has previously been shown that both the 8 and 9 Leu mutants are SecA dependent [8]. Since the 9 Leu precursors can still translocate it would appear that the doxorubicin has not incapacitated the Sec dependent translocation machinery. The binding of doxorubicin to biological membranes has also been shown to affect a number of membrane associated processes including electron transfer [13] and this may therefore affect the proton-motive force (pmf) across the membrane. Both the 8 and 9 Leu mutants have been shown to undergo pmf dependent translocation hence, since the 9 Leu precursors translocate, alteration in pmf can not be the reason for the inhibition of translocation in the case of the 8Leu precursors. A feasible explanation for the observed inhibition of PG dependent translocation would be that the doxorubicin is interacting with anionic lipid and thus preventing the lipid participating in the translocation reaction.

Polylysine also has the potential to interact with anionic lipid and has also been shown to have a inhibitory affect on the PG dependent translocation of wild type prePhoE [9] hence we wished to observe whether this was also the case for the OmpF-Lpp mutants. Vesicles were therefore pre-incubated with polylysine prior to the translocation assay. As can be seen in Fig. 3 the PG dependent translocation reaction undertaken by the 8 Leu mutants was inhibited yet the PG independent translocation of the 9 Leu mutants was able to continue irrespective of the level of polylysine present. Furthermore the level of translocation inhibition seen with these mutants is of a similar magnitude to that observed in the case of the wild type precursor protein prePhoE [9] thus implying that the inhibition of PG dependent translocation in this manner is independent of the protein studied but is due to the interaction of polylysine with a key element in the translocation pathway. We believe that this element is the anionic lipid (PG) and that the interaction of membrane interactive, positively charge compounds with the lipid headgroup prevents the participation of this lipid species in translocation.

In summary, both polylysine and doxorubicin are able to inhibit the PG dependent translocation of the 8 Leu OmpF-Lpp signal sequence mutants but not the PG

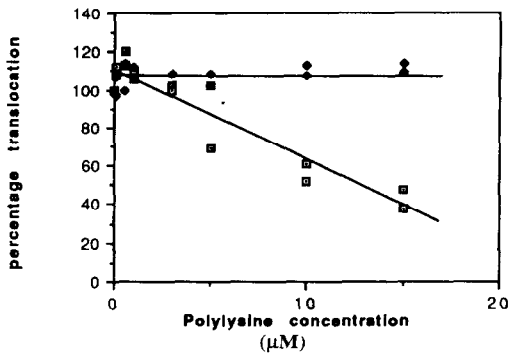

Fig. 3. Effect of polylysine on the translocation of 2K8L and 2K9L. Translocation assays were performed using wild type membrane vesicles (MRE600). The translocation efficiencies of 2K8L (□) and 2K9L (◆) were observed in the presence of varying concentrations of polylysine. 100% represents wild type translocation. Samples were analysed by tricine SDS-PAGE and fluorography and quantified by scintillation counting. All points are the mean of two to three experiments.

independent translocation of the 9 Leu mutants. Since the only known variables in the translocation of these two series of mutants are the PG requirement and the requirement for positive charges at the amino terminus of the signal peptide it is likely that inhibition of the PG dependent pathway is due to the electrostatic interaction of the positively charged compounds with the anionic lipid head group. It is therefore probable that the signal sequence positive charges are directly interacting with the lipid in an electrophoretic manner during the course of protein translocation.

Alternatively it may be possible that the signal sequences are differentially interacting with the Sec machinery thus altering the requirement of the machinery for interaction with PG. The fact that directly comparable levels of translocation inhibition have been seen for prePhoE implies that this PG requirement is not only specific to these mutants.

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