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Assembly of eukaryotic class III (N-out, C-in) membrane proteins into the *Escherichia coli* cytoplasmic membrane

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Class III membrane proteins lack cleavable signal peptides but adopt an N-out, C-in topology with respect to their native membranes. We have analysed the fate of two eukaryotic class III plasma membrane proteins, human erythrocyte glycophorin C and influenza A virus M2 protein, in *Escherichia coli*. The N-terminal domains of both proteins were efficiently localised to the extracytoplasmic side of the bacterial cytoplasmic membrane. When β-lactamase was fused to the C-terminus of glycophorin C it was localised to the cytoplasm, and protease treatment of spheroplasts caused a reduction in size of the fusion protein consistent with glycophorin C adopting its native topology in *E. coli*.

Glycophorin C (human); M2 protein (influenza A virus); β-Lactamase; Membrane protein topology; Fusion analysis; *E. coli*

1. INTRODUCTION

Simple membrane proteins fall into three topologically distinct classes [1]. Class I membrane proteins possess an N-terminal signal peptide which directs translocation of the polypeptide across the membrane until halted by a 'stop–transfer' sequence. The signal peptide is cleaved from the pre-protein producing a mature protein with an N-out, C-in topology. Class II membrane proteins have an N-terminal uncleaved signal peptide which lodges in the membrane and translocates all downstream sequences, producing the N-in, C-out topology. Class III membrane proteins lack cleavable signal peptides yet their N-termini are translocated across the membrane. Translocation is dependent upon a downstream 'start–stop transfer' sequence and results in an N-out, C-in topology.

Gene fusion (topology) analysis has been used to demonstrate that representative class I and class II eukaryotic plasma membrane proteins expressed in *Escherichia coli* insert topologically correctly into the bacterial cytoplasmic membrane [2,3]. We have now analysed the fate in *E. coli* of two eukaryotic class III membrane proteins, human glycophorin C and the influenza virus M2 protein. We show that the N-terminal domains of both are localised to the *E. coli* periplasm. Moreover, by fusing β-lactamase towards the C-terminus of glycophorin C and determining the transmembrane (TM) disposition of the fusion protein, we deduce that glycophorin C assembles topologically correctly into the bacterial cytoplasmic membrane. Hence *E. coli* provides a suitable environment for the extracytoplasmic localisation of the N-termini of these eukaryotic class III membrane proteins.

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2. MATERIALS AND METHODS

2.1. *Subcloning the glycophorin C coding region for bacterial expression and fusion of β-lactamase to its C-terminal domain*

pYZ4 encodes a modified *LacZ* α-peptide with an *Nco*I site flanking its initiation codon, under the control of the *lac* uv5 promoter [3]. Insertion of an intact coding region between the *Nco*I site and another restriction site in the multiple cloning region ensures its direct expression. Linker tailing [4] was used to insert the first four codons of the glycophorin C gene, which include a unique *Sal*I site, between the *Nco*I and *Hin*dIII sites of pYZ4 using the oligonucleotides dCATGTGGTCGACGTGATA and dAGCTTATCACGCGTCGACCA. A cDNA encoding glycophorin C lacking its three C-terminal codons was removed from BET1 [5] as a *Sal*I-to-*Sca*I fragment and subcloned into pYZ4, thus creating pLH11. The mature β-lactamase coding region of pYZ5 [3] was fused in-frame to the C-terminally truncated gene by cutting pLH11 with *Xba*I, end-filling (with dCTP and dTTP only), endtrimming and cutting with *Eco*RI and ligating to a *Pvu*II–*Eco*RI β-lactamase fragment from pYZ5, making pLH12. A construct containing the entire coding region of glycophorin C (pEH5) was constructed by ligating a *Sal*I–*Sma*I fragment from BET1 to pYZ4. All fusion junctions were verified by DNA sequencing using the Sequenase II system (United States Biochemical Corp.) according to the manufacturer's instructions.

2.2. *Subcloning the influenza M2 coding region for bacterial expression*

The polymerase chain reaction was used to amplify an M2 cDNA and simultaneously introduce a *Bsp*HI site spanning its initiation codon using primers dAATCATGAGCCTTCTGACCGA and dTTTACTCCAGCTCTATGCTGA. The amplified fragment was first cloned into an M13mp18 T-vector [6] then subcloned into *Nco*I and *Eco*RI cut pEH1 (a derivative of pYZ4 that contains a phage T7 promoter in addition to the *lac* uv5 promoter; E.S.H. and J.K.B-S., unpublished data) as an *Bsp*HI–*Eco*RI fragment, thus creating LHB1.

2.3. *Synthesis of the membrane proteins in* E coli *and production and protease-treatment of spheroplasts*

Plasmids were transformed into *E. coli* strain 5K where the cloned gene products are synthesised constitutively. Cultures were grown to log phase at 37°C with constant shaking. Cells were harvested by centrifugation and the pellet resuspended in 1/80th original volume of 25% (w/v) sucrose; 30 mM Tris-Cl, pH 8.0; 1 mM PMSF then made 10 mM EDTA. 40 μM PMSF. Spheroplasts were produced by adding 1/25th vol. lysozyme (10 mg/ml). Plasmid pLHB1 was also transformed into strain BL21(DE3) which encodes T7 RNA polymerase under the control of the *lac* promoter [7]. Synthesis of M2 protein was induced by the addition of 1 mM IPTG to log phase cells. Cells were grown for a further hour and then pelleted by centrifugation. Since the outer membranes of BL21(DE3) cells were resistant to permeabilisation using EDTA and lysosyme, spheroplasts were produced using polymyxin B sulphate and lysosyme as described in [8].

Aliquots of spheroplasts were treated either with proteinase K (2.5 mg/ml final concentration) by incubating at room temperature for 5 min then stopping digestion with 5 mM PMSF, or with TPCK-treated trypsin (0.75 μg/ml) at 4°C for 60 min, and digestion stopped by adding the inhibitors aprotinin (1 TIU/ml) and PMSF (1 mM). Further aliquots were treated in an identical fashion except that the protease addition step was omitted.

2.4. *Protein gels and Western blotting*

Samples were lysed by repeated cycles of freeze–thawing and then $MgCl_2$ (10 mM) and DNase (10 μg/ml) were added. Samples were subjected to SDS-PAGE [9] and transferred to nitrocellulose. Primary antibodies (diluted 1:1,000) were against β-lactamase (Northumbria Biologicals Ltd.), a peptide corresponding to the N-terminal 19 amino acids of glycophorin C (S. High, University of Manchester, and M Tanner, University of Bristol) or the N-terminus of flu M2 protein [10] An alkaline phosphatase-linked secondary antibody (1:1000 dilution; Sigma Chemical Co.) was used Cross-reacting proteins were detected by incubating the nitrocellulose in 100 mM NaCl, 5 mM $MgCl_2$, 100 mM Tris-Cl, pH 9.5, containing nitroblue tetrazolium (0.033% (w/v)) and bromochloroindoyl phosphate, toluidine salt (0.017% (w/v)). The reaction was stopped by the addition of 20 mM EDTA.

3. RESULTS AND DISCUSSION

Glycophorin C is a 128 amino acid protein found in erythrocytes which adopts an N-out, C-in topology within the membrane with its N-terminal 58 amino acids being extracytoplasmic [5,11]. Cells of 5K(pEH5) synthesising glycophorin C were spheroplasted and incubated with proteinase K. SDS-PAGE and Western blotting using an antibody directed to the N-terminal 19 amino acids of glycophorin C revealed that the N-terminus was localised to the periplasmic side of the *E. coli* cytoplasmic membrane (Fig. 1a).

To verify the N-out, C-in topology of glycophorin C in *E. coli*, β-lactamase was fused to the C-terminus of glycophorin C and spheroplasted cells of 5K(pLH12) were treated as before. As expected, the N-terminal domain of the glycophorin C portion was extracytoplasmic (Fig. 1a), and proteinase K treatment resulted in an apparent 7.5 kDa size reduction, as detected using β-lactamase antibody (Fig. 1b). This is compatible with all but the predicted 7 kDa N-terminal domain of glycophorin C being protected from proteolysis. In a control reaction a cytoplasmic β-lactamase fusion protein, pYZ11/blaM11 [3] remained proteinase K resistant indicating that the cytoplasmic membrane had not been permeabilised (Fig. 1c).

When cells of 5K(pLH12) were inoculated at high density onto L-agar plates containing increasing concentrations of ampicillin, the minimum inhibitory concentration of ampicillin was 150 μg/ml, a value that is within the range encountered for other β-lactamase fusion proteins the detection of which requires Western blotting. However, when cells of 5K(pLH12) were inoculated at low density, so that they were *individually* exposed to the antibiotic, they failed to form colonies on agar containing 5 μg/ml ampicillin. The inability of the fusion protein to protect individual cells against ampicillin-induced lysis is an indicator of a cytoplasmic location for the β-lactamase moiety, and hence the C-terminal domain of glycophorin C (see [12,13] for reviews).

The influenza A virus M2 protein is a 97 amino acid-long class III membrane protein, which assembles into the plasma membrane of infected cells with its N-terminal 20 (or so) residues forming the extracellular domain [14]. pLHB1 was transformed into 5K, but constitutive synthesis of the M2 protein from the *lac* uv5 promoter yielded no detectable cross-reacting product using the M2 antibody for the N-terminus (data not shown). Therefore pLHB1 was transformed into strain BL21(DE3), and T7 promoter-mediated expression of the M2 gene was induced. When cells were converted to spheroplasts and trypsin treated this resulted in the loss of the N-terminal M2 epitope, indicating that this domain achieves an extracytoplasmic location in *E. coli* (Fig. 1d).

The data presented here demonstrate that when glycophorin C or the M2 protein are synthesised in *E. coli*, their N-termini are on the periplasmic side of the cytoplasmic membrane. In the case of glycophorin C, further analysis demonstrates that it adopts its normal class III (N-out, C-in) topology in *E. coli*.

Since protease treatment of spheroplasts removed all of the product that cross-reacted with the N-terminus specific antibodies (Fig. 1a and d) this suggests that few if any molecules of glycophorin C or the M2 protein assemble into the *E. coli* cytoplasmic membrane with an inverted topology. The glycophorin C::β-lactamase fusion protein conferred no protection on cells against ampicillin-induced lysis, providing a further indication that glycophorin C assembles efficiently into the *E. coli* cytoplasmic membrane. The N-terminal domain of glycophorin C is normally extensively glycosylated, but since glycosylation does not occur in *E. coli* we can conclude that such modification is not required for correct assembly.

The system described here allows the expression of eukaryotic class III TM proteins in *E. coli* while maintaining their correct topology relative to the cytoplasmic membrane. The criteria that determine the translocation competence of N-terminal domains of class III

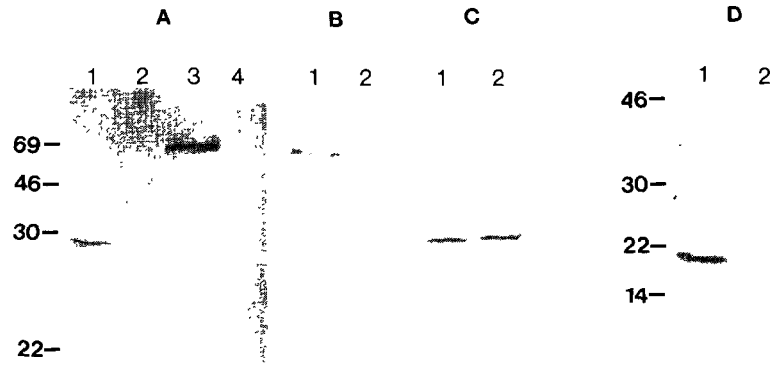

Fig. 1. TM organisation of glycophorin C and M2 protein in *E. coli*. Cells synthesising glycophorin C, the glycophorin C::β-lactamase fusion protein, the M2 protein or the control pYZ11/blaM 11 were spheroplasted, protease treated, and subjected to SDS-PAGE and Western blotting as described in section 2. Proteins were run on 12.5% (w/v) polyacrylamide gel (a–c) or a 15% (w/v) polyacrylamide gel (d) and detected using antibody to the N-terminus of glycophorin C (a), β-lactamase (b and c), or the N-terminus of M2 protein (d). (a) Lanes 1 and 2, glycophorin C minus and plus proteinase K. Lanes 3 and 4: glycophorin C::β-lactamase minus and plus proteinase K. (b) Lanes 1 and 2, glycophorin C::β-lactamase minus and plus proteinase K. (c) Lanes 1 and 2, control pYZ11/blaM11 minus and plus proteinase K. (d) Lanes 1 and 2, M2 protein minus and plus trypsin. The molecular weights (in kDa) of Rainbow marker protein standards (Amersham) are shown.

proteins are relatively unexplored. The recent findings that two TM proteins (Unc-5 and Neu differentiation factor) with exceptionally large extracytoplasmic N-terminal domains probably lack cleavable signal peptides [15,16], and that the extracellular localisation of the relatively short N-terminal domain of the human β_2-adrenergic receptor may be naturally inefficient [17], provide further impetus for examining these criteria. Such issues can be easily and quickly addressed using model class III membrane proteins in *E. coli*. It is known that the assembly of the M2 protein and glycophorin C into eukaryotic membranes is signal recognition particle (SRP) dependent ([18] and S. High, personal communication). It will be interesting to determine whether SRP of *E. coli* [19], or other bacterial chaperones, are required for the assembly of these proteins into the bacterial cytoplasmic membrane.

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