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Overcoming the toxicity of membrane peptide expression in bacteria by upstream insertion of Asp-Pro sequence ☆

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Abstract

Transmembrane (TM) peptides often induce toxic effects when expressed in bacteria, probably due to membrane destabilization. We report here that in the case of the TM domains of hepatitis C virus (HCV) E1 and E2 envelope proteins, which are both particularly toxic for the bacteria, the insertion of the Asp-Pro (DP) sequence dramatically reduced their toxicities and promoted their expressions when produced as glutathione *S*-transferase (GST) GST-DP-TM chimeras. Subcellular fractionation showed that these chimeras co-sediment with the membrane fraction and contain active GST that could be solubilized with a mild detergent. Surprisingly, immuno-gold electron microscopy clearly showed that such chimeras are not localized in the membrane but in the cytosol. We thus postulate that they likely form proteo-lipidic aggregates, which prevent the bacteria from toxicity by sequestering the TM part of the chimeras. The reduction of toxicity in the presence of the Asp-Pro sequence is possibly due to Asp's negative charge that probably disadvantages the binding of the TM peptides to the membrane. In addition, the structural features of Pro residue could promote the formation of chimera aggregates.

Keywords: Bacterial expression; Transmembrane domain; Membrane peptide; Hepatitis C virus glycoproteins E1 and E2

1. Introduction

Membrane proteins represent about 30% of all proteins to be encoded by genomes [1,2]. There is growing interest in these proteins due to their wide spectrum of structural and functional roles, such as solute transport [3], signal transduction and communication between compartments, but also viral envelope assembly or membrane fusion. Their membrane localization is ensured by the presence of one or more hydrophobic stretches of 20–25 residues [4,5], which are generally folded in α-helices [4–6] but in some cases in β-sheets [7,8]. Structural information contained within these transmembrane (TM) segments in terms of three-dimensional structure, orientation, shape and packing is critical for further understanding of their function. However, the determination of their structure by crystallography or NMR remains a bottleneck due to the difficulties encountered in producing large amounts of membrane peptides and proteins [9,10].

Abbreviations: Amp, ampicillin; BSA, bovine serum albumin; DM, β-D-dodecyl maltoside; DP, aspartate-proline sequence; EDTA, ethylendiaminetetraacetic acid, Na_4; ER, endoplasmic reticulum; GST, glutathione *S*-transferase; GSTkt, glutathione *S*-transferase expressed from the pGEXKT plasmid; HCV, hepatitis C virus; IPTG, isopropyl-1-thio-β-D-galactoside; Kan, kanamycin; LB, Luria Bertani culture medium; OD, optical density; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; PBS, phosphate buffered saline; PMSF, phenylmethylsulfonyl fluoride; rpm, rotations per minute; SDS, sodium dodecyl sulfate; TM, transmembrane; TME1 and TME2, HCV E1 and E2 envelope glycoprotein TM domains

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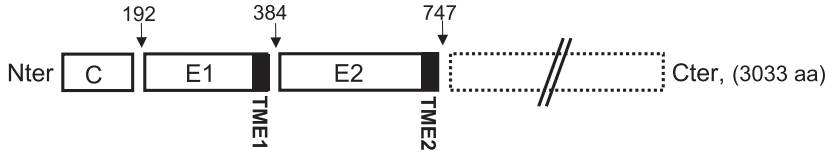
Due to their limited size, TM segments are often produced by chemical synthesis, though their hydrophobic nature makes their successful synthesis uncertain in terms of purity and yield. This is mainly due to the propensity of hydrophobic peptides to aggregate and the difficulties encountered in maintaining their solubility. Their purification by conventional means (e.g. RP-HPLC) is problematic as they tend to interact irreversibly with the stationary phase of chromatographic columns [11]. The addition of charged, preferentially basic residues to the hydrophobic peptide ends has been described to reduce these effects (cf. Melnyk et al. [12] and references therein). For longer hydrophobic peptides, chemical synthesis remains possible, but only in a limited number of cases [13]. NMR is generally used for three-dimensional structure determination of short membrane domains [14], but this often requires uniform ^{15}N and/or ^{13}C labeling which is very expensive due to the price of labeled amino acids. A cheaper alternative is the overproduction of these peptides in bacteria using ^{15}N and/or ^{13}C labeled growth medium. However, membrane proteins often induce toxic effects which can lead to the death of the microorganism [15,16], possibly due to membrane destabilization and leakage or pore formation. Different systems have been developed for optimizing the expression of membrane proteins, either by modifying the strain [15,17], or reducing toxicity by the insertion of a leader peptide [18], or by controlling their expression [19,20]. When produced, hydrophobic peptides may accumulate in the membrane fraction or in the form of inclusion bodies, which can then be further purified. A better understanding of recombinant membrane peptide expression in bacteria would be helpful in overcoming their toxicity.

Hepatitis C virus (HCV) is a major cause of liver diseases such as cirrhosis and hepatocellular carcinoma. This virus contains a 9500-base-long positively stranded RNA [21] which encodes for a single polyprotein at the level of the ER membrane. This polyprotein is further



TME1: $_{348}$**IAGAHWGVLAGIAYFSMVGNWAKVLVVLLLFAGVDA**$_{383}$

TME2: $_{717}$**EYVVLLFLLLADARVCSCLWMMLLISQAEA**$_{746}$

TME1 expressed with pT7-7,

- DP: M_{348}IAGAHWGVLAGIAYFSMVGNWAKVLVVLLLFAGVDA$_{383}$
+ DP: MDP$_{348}$IAGAHWGVLAGIAYFSMVGNWAKVLVVLLLFAGVDA$_{383}$

TME2 expressed with pT7-7,

- DP: M_{717}EYVVLLFLLLADARVCSCLWMMLLISQAEA$_{746}$
+ DP: MDP$_{717}$EYVVLLFLLLADARVCSCLWMMLLISQAEA$_{746}$

TME1 expressed with pGEXKT,

- DP: $_{1}M...{}_{223}GGGGGLVPRGS_{233}$-$_{348}$IAGAHWGVLAGIAYFSMVGNWAKVLVVLLLFAGVDA$_{383}$
+ DP: $_{1}M...{}_{223}GGGGGLVPRGS_{233}$-DP$_{348}$IAGAHWGVLAGIAYFSMVGNWAKVLVVLLLFAGVDA$_{383}$

TME2 expressed with pGEXKT,

- DP: $_{1}M...{}_{223}GGGGGLVPRGS_{233}$-$_{717}$EYVVLLFLLLADARVCSCLWMMLLISQAEA$_{746}$
+ DP: $_{1}M...{}_{223}GGGGGLVPRGS_{233}$-DP$_{717}$EYVVLLFLLLADARVCSCLWMMLLISQAEA$_{746}$

Fig. 1. HCV polyprotein processing, and amino acid sequences of E1 and E2 envelope glycoprotein TM domains and GST chimeras expressed in the present study. Schematic representation of the HCV polyprotein. After translation, processing of the N-terminal region of the polyprotein by ER signal peptidase generates core protein (C) and the envelope proteins E1 and E2. Arrows indicate the cleavage sites and the numbers indicate the first N-terminal amino acid of the generated proteins (numbered according to the positions of amino acid residues in the HCV polyprotein of genotype 1a, strain H). Ectodomain and TM domains of E1 and E2 are symbolized by a tandem of open and closed rectangles, respectively. The dotted rectangle symbolizes the rest of the polyprotein including p7 and NS3 to NS5B proteins. The amino acid sequences of E1 and E2 C-terminal regions containing their respective TM domains (underlined residues) correspond to those expressed in this work. The numbers indicated at sequence edges correspond to the position of residues in the polyprotein (HCV genotype 1a; EMBL access number: #D00831 and #M67463 for TME1 and TME2, respectively). Sequences of the proteins and GST chimeras expressed in the present work are displayed. Residues belonging to the expression plasmid are in italics. Sequence of GSTkt is indicated in a box and its C-terminal region is detailed.

processed by cellular and viral proteases to give at least 10 proteins (for a review, see Ref. [22]). Among these, the two glycoproteins E1 and E2 are responsible for viral envelope formation. Processing of these proteins is achieved by signal peptidase cleavage in the endoplasmic reticulum (ER) lumen (Fig. 1). E1 and E2 are type I TM proteins with N-terminal ectodomains of about 160 and 360 amino acids, respectively, and a short C-terminal TM domain of approximately 30 amino acids (Fig. 1). Both E1 and E2 TM domains are formed by two stretches of hydrophobic amino acids separated by a short polar segment containing fully conserved charged residues [23]. After signal peptidase cleavage, each TM domain forms a single TM helix as a result of a dynamic process of C-terminal stretch reorientation [24,25]. E1 and E2 TM domains are involved during different steps of virus formation, acting as translocation re-initiation signals for the downstream coded proteins and promoting E1–E2 heterodimer formation [26]. They are also probably involved in fusion complex formation for virus cell entry. Structural analysis of both E1 and E2 TM domains is thus essential to provide a framework for the molecular understanding of these mechanisms and which could permit the identification of novel targets for antiviral intervention. However, the expression of full-length E1 and E2 proteins or their domains using eukaryotic heterologous expression systems [27] yields insufficient amounts of protein for structural investigations. Attempts at producing the full-length E1 protein in *E. coli* or in *sf*9 insect cells using the baculovirus system [28–30] have had limited success, due to the toxicity associated with expression of the protein, or more precisely that of its C-terminal hydrophobic domain. No attempts to express E2 in bacteria have so far been reported.

In this report, we describe that the expression of the TM domains of HCV E1 (TME1) and E2 (TME2) in *E. coli*, either alone or fused with glutathione *S*-transferase (GST), was not possible due to very high associated toxicity. Surprisingly, however, the insertion of an Asp-Pro sequence between the GST and TME1 or TME2, added for chemical cleavage purposes, reduced TME1 and TME2 toxicity and promoted their overexpression as GST chimeras. Cell fractionation experiments showed that a significant amount of the GST chimeras sedimented with the membrane fraction in an active GST form that could be further solubilized with a mild detergent. At the ultrastructural level, however, these chimeras were immuno-localized only in the cytosol. These data suggest the existence of micellar-like aggregates and proteo-lipidic aggregates that prevent the membrane peptide to reach the membrane and consequently confer protection against toxicity. The reduction in toxicity due to the presence of an Asp-Pro sequence is tentatively attributed to the negative charge on the Asp residue that probably reduces the propensity of TM domains binding to the membrane and/or to the promotion of micellar forms of aggregates possibly facilitated by the presence of Pro residues.

2. Experimental procedures

2.1. Materials

Oligonucleotides were synthesized by EUROBIO. The Qiaprep kit for DNA plasmid preparation was from QIAGEN. The BigDye™ Terminator Cycle ABI PRISM® for DNA sequencing was from Applied Biosystems. *E. coli* BL21(DE3) and BL21(DE3)[pLysS] strains were purchased from Stratagene. The C41 and C43 [15] (BL21(DE3)) *E. coli* strains were kindly provided by Dr. Bruno Miroux (Faculté de Médecine Necker-Enfants-Malades, CNRS-UPR 9078, 156 rue de Vaugirard, 75730 PARIS CEDEX 15). DNA restriction and modification enzymes were from New England Biolabs. Precision protein standards, Kaleidoscope prestained standards, miniprotean 3 and its transblot module, GS-700 and Molecular Analyst were from Bio-Rad. The pCR®T7 topo TA plasmid was from Invitrogen. The Vibracell 72408 was from Fisher Bioblock Scientific. The pET32a+ plasmid was from Novagen. K38 *E. coli* strain, pT7-7 and pGP1-2 plasmids [31] were kindly provided by Prof S. Tabor (Department of Biological Chemistry, Harvard Medical school, Boston, Mass, USA). The pGEXKT plasmid [32] was kindly provided by Prof. Dixon (Department of Biological Chemistry, University of Michigan Medical School, Ann Arbor, USA). Bacterial culture products were from Difco Laboratories and *N*-dodecyl-β-D-maltoside (DM) was from Anatrace Inc. Immobilon-P membranes were from Millipore and the Anti-GST antibody GST(Z-5):sc-459 was from Santa Cruz Biotechnology, Inc. The ECL kit was from Amersham Biosciences and gold particles conjugated to anti-rabbit IgG were from British Biocell. LR white resin was from Electron Microscopy Sciences. Other products were purchased from Sigma-Aldrich Corp.

2.2. Strains

Epicurian Coli® BL21(DE3): *E. Coli* B F^- *dcm ompT hsdS*($r_B^- m_B^-$) *gal* λ(DE3), Epicurian Coli® BL21(DE3)[pLysS]: *E. coli* BL21(DE3)[pLysS Cam^r]. *E. coli* K38 (HfrC λ) is described by Tabor [31]. C41 and C43 *E. coli* strains were obtained from BL21(DE3) and described by Miroux and Walker [15].

2.3. Plasmid constructs

Genes encoding the E1 and E2 TM domains (TME1 and TME2) were generated de novo by polymerase chain reaction (PCR) [33] using optimized codons for *E. coli* expression [34]. Subcloning for the different plasmids was done using a set of two long and two short oligonucleotides as displayed in Table 1. The corresponding TME1 and TME2 amino acid sequences are shown in Fig. 1. All constructions were optimized by using vector NTI 6.0 software.

Table 1
Oligonucleotides used to generate and clone TME1 and TME2 coding sequences

TME1 gene synthesis and cloning:		
Synthesis	OL11(+) 5′-atgc*catatg*atcgctggtgctcactggggtgttctggctggtatcgcttacttctctatggttggtaactggg OL12(−) 5′-gcat*atcgat*ctaagcgtcaacaccagcgaacagcagcagaacaaccagaactttagcccagttaccaaccatagagaa	
	Without DP sequence:	With DP sequence:
Sub-cloning in pT7-7	OL13(+) 5′-ggga*atgccat*atgatcgctggtg	OL113(+) 5′-*gaattc*ctaagcgtcaacaccagc
	OL14(−) 5′-gcat*atcgat*ctaagcgtcaaca	OL114(−) 5′-cg*catatg*gacccgatcgctggtgct
Sub-cloning	OL15(+) 5′-*ggatcc*atggaatacgttgttc	OL17(+) 5′-*ggatcc*gacccgatggaatacgttgttc
	OL16(−) 5′-*gaattc*ctaagcttcagcctgag	OL16(−)
TME2 gene synthesis and cloning:		
Synthesis	OL21(+) 5′-*catatg*gaatacgttgttctgctgttcctgctgctggctgacgctcgtgtttgctcttgcctgtggat OL22(−) 5′-*aagctt*aagcttcagcctgagagatcagcagcatcatccacaggcaagagcaaacac	
	Without DP sequence:	With DP sequence:
Sub-cloning in pT7-7	OL23(+) 5′-*catatg*gaatacgttgttc	OL213(+) 5′-cag*aattc*ctaagcttcagcctgagag
	OL24(−) 5′-*aagctt*aagcttcagcctgagagatcag	OL214(−) 5′-cg*catatg*gacccggaatacgttgttc
Sub-cloning in pGEXKT	OL25(+) 5′-*ggatcc*gaatacgttgttc	OL27(+) 5′-ggatcc*gacccg*gaatacgttgttc
	OL26(−) 5′-*gaattc*ttaagcttcagcctgagagatcag	OL26(−)

Constructions and designs of the oligonucleotides were carried out with vector NTI 6.0 and Oligo 5.0 softwares. Starting codon ATG is underlined twice. Underlined bases correspond to the hybridizing region between each couple of long oligonucleotides. Restriction sites are in italics.

Cloning into pT7-7 [31] without insertion of the DP sequence was achieved by generating PCR fragments from each synthetic DNA with OL13 and OL14 primers for TME1 and OL23 and OL24 primers for TME2 (Table 1). Fragments were subcloned into the pCR®T7 topo TA plasmid, then extracted using *Nde*I and *Cla*I restriction enzymes for TME1 and *Nde*I and *Hin*dIII restriction enzymes for TME2, and finally cloned into pT7-7 using the same sites to yield plasmids pT7-7-TME1 and pT7-7-TME2.

Cloning into pGEXKT [35], in frame with the 3′ end of the gene coding for GSTkt and with or without the sequence encoding the Asp-Pro, was achieved by generating PCR fragments from each synthetic DNA with OL15 (−DP) or OL17 (+DP) and OL16 (TME1) and OL25 (−DP) or OL27 (+DP) and OL26 (TME2) primers (Table 1). Fragments were subcloned into the pCR®T7 topo TA plasmid and then cloned into pGEXKT using *Bam*HI and *Eco*RI restriction enzymes to yield plasmids pGEXKT-TME1, pGEXKT-TME2, pGEXKT-$_{DP}$TME1 and pGEXKT-$_{DP}$TME2.

Cloning into pT7-7 [31] with the insertion of the DP sequence was achieved by generating PCR fragments from plasmids pGEXKT-$_{DP}$TME1 and pGEXKT-$_{DP}$TME2 with OL113 and OL114 primers for TME1 and OL213 and OL214 primers for TME2 (Table 1). Fragments were cloned into pT7-7 using *Nde*I and *Eco*RI restriction enzymes to give plasmids pT7-7-$_{DP}$TME1 and pT7-7-$_{DP}$TME2. All the produced proteins are summarized in Fig. 1.

2.4. Expression tests

Expression with pT7-7 constructs was carried out as described [31] after transformation of K38[pGP1-2], BL21(DE3) or BL21(DE3)[pLysS] bacteria. When possible, about 10 clones were picked from plates and tested for expression. Bacteria were diluted in 5 ml of Luria Bertani culture medium (LB) supplemented with ampicillin (and kanamycin for K38[pGP1-2]), then grown until saturation at 37 °C and 200 rpm. Cultures were then diluted 1/10 in the same medium and grown for 1 h before induction, either by increasing the temperature to 42 °C for 15 min for the K38 strain, or by adding 1 mM isopropyl-1-thio-β-D-galactoside (IPTG) for the BL21(DE3)-type strains. Expression was continued for 3–6 h at 37 °C and growth of each culture was followed by periodic checking of the $OD_{600\ nm}$. After a 3-h expression, a volume equivalent to 0.1 $OD_{600\ nm}$ was withdrawn and pelleted. The pellet was suspended in 50-μl water then 50-μl lysis buffer (see Section 2.8), followed by mixing and heating for 1 min at 100 °C before analyzing 10 μl by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

2.5. Expression at low temperature

Bacteria transformed with pGEXKT, pGEXKT-$_{DP}$TME1 or pGEXKT-$_{DP}$TME2 were grown in LB medium containing 50 μg/ml ampicillin, at 37 °C and 200 rpm, until a cell density of 2.0 $OD_{600\ nm}$/ml was reached. The cultures were then cooled on ice to quickly decrease the temperature to 20 °C and then incubated for at least 30 min at 20 °C before to induce them by adding 0.5 mM IPTG. Expression was carried out overnight (15–17 h) at 20 °C and 200 rpm. Cell density was estimated (~ 5–6 $OD_{600\ nm}$/ml) and bacteria were harvested by centrifugation at $5000 \times g_{av}$ (6750 rpm) in a JA10 Beckman rotor (J2-MC Beckman centrifuge), for 15 min at 4 °C. Bacteria were washed in the

same volume of cold water and centrifuged again. The pellet was suspended in buffer A (25 mM Tris–Cl pH 7.0, 5 mM $MgCl_2$, 5 mM β-mercaptoethanol, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF)) to a final density of ~ 100 $OD_{600\ nm}$/ml and either stored on ice for immediate use or frozen in liquid N_2 and stored at − 80 °C.

2.6. Cellular fractionation

Each bacterial suspension was poured into a 50-ml beaker and cells were disrupted with a Vibracell 72408 sonifier equipped with a 10-mm-diameter probe. Sonication was carried out for 1 min by ON/OFF pulses of 0.5 s and by setting the generator to 30% of maximal power. The suspension was maintained below 10 °C with ice. Disruption was continued until there was a 10-fold decrease in cell density, which generally took about 7 min. An aliquot of the suspension was withdrawn (“bacteria” in Fig. 4A), and unbroken material and inclusion bodies were discarded by centrifugation at 5000 × g_{av} (6750 rpm) in a JA10 Beckman rotor (J2-MC Beckman centrifuge), for 15 min. The supernatant (referred to as “bacterial extract”, Fig. 4) was centrifuged at 100,000 × g_{av} (36,000 rpm) in a Ti45 Beckman rotor (L8-M Beckman centrifuge), for 120 min at 10 °C. The membrane-rich pellet is referred to as the “membranous fraction”, and the supernatant that contained the soluble material is referred to as the “soluble fraction” (see Fig. 4). The pellet (“membranous fraction”) was suspended in 5–6 ml of buffer B (20 mM Tris–Cl pH 7.0, 300 mM sucrose, 5 mM β-mercaptoethanol, 1 mM EDTA), then frozen in liquid nitrogen and stored at − 80 °C.

2.7. Solubilization of membrane proteins

Solubilization of the “membranous fraction” was performed using 2-mg protein aliquots diluted in a final volume of 1-ml buffer B with the addition of 6-mg β-D-dodecyl maltoside/30 μl (DM/protein ratio = 3). Solubilization was carried out for 30 min at 20 °C, followed by centrifugation at 10 °C for 30 min at 100,000 × g_{av} (50,000 rpm) with a TLA100 rotor in a TL100 Beckman centrifuge.

2.8. Protein electrophoresis

Aliquots of 20 μl containing 0.1- to 120-μg protein were mixed with an equal volume of lysis buffer containing 100 mM Tris–Cl pH 8.0, 1.4 M β-mercaptoethanol, 140 mM SDS, 5 mM EDTA, 8 M Urea and 0.72 mM bromophenol blue [36]. Samples were heated at 100 °C for 1 min, cooled and then loaded, either on 14% polyacrylamide gels prepared and treated as described, either by Laemmli [37] for protein of MW higher than 10 kDa (SDS-PAGE), or on 16.5% tricine-polyacrylamide gels as described by Schagger and von Jagow [38], to separate peptides (tricine-PAGE). Electrophoresis was carried out with a Bio-Rad miniprotean 3 device.

2.9. Protein estimation

Protein concentrations were measured by the bicinchoninic acid procedure [39] in the presence of 17 mM SDS.

2.10. Western blotting

After SDS-PAGE, gels were incubated for 5 min in cold CAPS buffer (10 mM CAPS-NaOH pH 11.1, 10% MeOH) and proteins were blotted onto Immobilon-P membranes with a Bio-Rad Protein 3 transblot module for 90 min at 150 V and 250 mA, in cold CAPS buffer. Blotting membranes were incubated for 30 min in 20 ml of PBST buffer (90 mM K_2HPO_4, 10 mM KH_2PO_4, pH 7.7, 100 mM NaCl, 0.2% Tween-20 (polyoxyethylenesorbitan monolaurate), containing 10% dry fatty acid-free milk. GST(Z-5):sc-459 polyclonal antibody (+HRP) was added to a 1/2000 dilution for 1 h. Blots were then washed three times for 10 min in 20 ml of PBST buffer and bands detected by chemoluminescence, using the ECL kit.

2.11. GST activity measurements

GST activity was measured spectrophometrically according to the procedure published by Habig et al. [40]. Measurements were carried out in 2-ml 100 mM KH_2PO_4 pH 6.5, 1 mM GSH, in a cuvette thermostated at 25 °C with stirring. As substrate, 1 mM of 1-chloro-2,4-dinitrobenzene was added and the reaction was started by the addition of 5 μl of sample and recorded at 340 nm for 300 s. Slopes were corrected for the value obtained with the control assay carried out without enzyme.

2.12. Immuno-electron microscopy

Bacterial pellets were fixed in 2.5% paraformaldehyde in 0.15 M cacodylate buffer pH 7.4, rinsed in buffer alone and then dehydrated in graded ethanol solutions. Sample preparations were done as detailed previously [41,42]. Briefly, progressive infiltration with the LR white acrylic resin and embedding was performed according to the manufacturer’s instructions. Polymerization was induced by incubation at 56 °C for 24 h. Silver to gold sections were mounted on formvar-coated nickel grids. Before immunolabeling, sections were hydrated in phosphate-buffered saline (PBS) and saturated in 1% BSA diluted in PBS. Grids were then floated for 1 h on drops containing the primary antibody against GST (diluted 1/50 in PBS-BSA), rinsed, and incubated with 10-nm gold particles conjugated with anti-rabbit IgG (British BioCell, Cardiff, UK) diluted 1/30 in PBS-BSA for 45 min. After extensive washing in PBS, samples were fixed with 2% glutaraldehyde, rinsed with water and dried. Sections were contrasted in methanolic uranyl acetate and lead citrate. Observations were performed with a Philips CM120 transmission electron microscope at the Centre de Technologie des Microstructures, Claude Bernard University, Lyon, France.

3. Results

3.1. Choice of limits of TME1 and TME2 domains

Since E1 and E2 envelope glycoproteins are synthesized in series in the HCV polyprotein, between the Core and P7 proteins (Fig. 1), the C-terminal ends of TME1 and TME2 are generated by signal peptidase cleavage during processing, at amino acids 384 and 747, respectively. Their N-terminal ends are less precisely known, but according to previous work [27], we have chosen to start them at residues 348 for TME1 and 717 (positions on the polyprotein) for TME2. The sequences of TME1 and TME2, shown in Fig. 1, correspond to those expressed in this work. In order to optimize the biosynthesis of these small domains of 36 and 31 residues for TME1 and TME2, respectively, the coding genes were generated by PCR using *E. coli* preferred codons (see Section 2).

3.2. Expression of TME1 or TME2 is highly toxic for E. coli

To test the expression of TME1 and TME2 alone, we first tried the system developed by Tabor [31] based on a T7 promoter and thermal induction as described in the original procedure (see Section 2). The first unusual feature was that the yield of transformation of the K38[pGP1-2] *E. coli* strain with the expression plasmids pT7-7-TME1 or pT7-7-TME2 was very low. In addition, transformed strains did not produce detectable amounts of peptides, at least at the Coomassie-staining level, as judged by electrophoresis carried out with tricine-PAGE [38] (not shown).

The same constructs were used to transform BL21(DE3) and BL21(DE3)[pLysS] strains in which the gene coding for the T7 RNA polymerase was integrated in the bacterial genome and induced by IPTG (see Section 2). The same transformation difficulties were observed for the BL21(DE3) strain. In contrast, it was possible to get transformed BL21(DE3)[pLysS] clones for which the small amount of T7 lyzozyme expressed by the pLysS plasmid efficiently repressed traces of T7 RNA polymerase expressed without induction. However, as shown in Fig. 2A, positive clones containing pT7-7-TME1 or pT7-7-TME2 stopped growing just after the addition of IPTG. No specific protein expression was observed on tricine-PAGE stained with Coomassie blue (Fig. 2B), whatever clones were tested. As indicated in Section 2, the expression experiments were carried out at 37 °C. Since reducing the temperature is known to promote the expression of membrane proteins [9,43], we also tried to reduce the temperature during the expression step, but this led neither to expression of the peptides nor reduction of the toxicity (not shown). These results demonstrate that the small amounts of TME1 or TME2 expressed at the very beginning of the induction were sufficient to kill the bacteria. This can also explain why the selection of clones from the K38[pGP1-2] or BL21(DE3) strains was so difficult. Indeed, expression in these strains is not as tightly controlled as that bearing pLysS.

3.3. The expression of TME1 and TME2 as GSTkt chimeras reduces their toxicity for E. coli

The expression of TME1 and TME2 fused to the C-terminus of GST was tested using the pGEXKT plasmid designed by Hakes and Dixon [32]. This plasmid contains a sequence coding for a linker of five glycine residues followed by the thrombin site at the C-terminus of GST. The GST protein expressed in the pGEXKT plasmid corresponds to GST-GGGGGLVPRGSPGIHRD and is referred to below as GSTkt. GSTkt-TME1 corresponds to the sequence GST-GGGGGLVPRGS-TME1, and GSTkt-TME2 corresponds to GST-GGGGGLVPRGS-TME2. Amino acid sequences of both chimeras are detailed in Fig. 1. Neither T7 RNA polymerase nor pLysS was necessary to test the expression of the GSTkt chimeras since pGEXKT carries the gene coding for GSTkt under the control of an IPTG-inducible promoter. Nevertheless, the BL21(DE3) [pLysS] *E. coli* strain was used for comparison purposes with the expression of TME1 and TME2 alone. It was possible to select positive clones after transformation and to test them for expression. When compared to the growth curve obtained for pT7-7 constructs (Fig. 2A), the induction of GSTkt-TME1 and GSTkt-TME2 expression by IPTG resulted in reduced toxicity, as shown in Fig. 2C. However, among about 20 clones tested for each construct, none expressed GSTkt-TME1 in detectable amounts and only one expressed a limited amount of the GSTkt-TME2 chimera (Fig. 2D). The latter was only produced in the form of inclusion bodies and all attempts at solubilization and refolding of GST failed (not shown), thereby preventing recovery of TME2 by thrombin cleavage.

We also carried out a series of experiments in which expression of chimeras was tested in the C41 and C43 BL21(DE3)-type *E. coli* strains isolated by Miroux and Walker [15] and found to be resistant to the toxicity induced by the expression of membrane proteins. Unfortunately, no expression was obtained with these strains, whatever the temperature. In addition, it was possible to isolate several resistant strains, but none of them expressed the chimeras.

3.4. Insertion of an Asp-Pro sequence between GST and TME1 or TME2 dramatically reduces the toxicity of TM domains and promotes GST chimera production

As described above, TME2 could not be cleaved from GSTkt-TME2 inclusion bodies by thrombin. An Asp-Pro sequence (DP) was thus introduced upstream to TME2 in the construct (see sequence in Fig. 1) to allow its release from GST by acidic chemical cleavage. The Asp-Pro bond is well known to be specifically cleaved in the presence of formic acid (70%) or diluted HCl (10 mM) [44], i.e., under conditions that do not require the native state of the GST chimera.

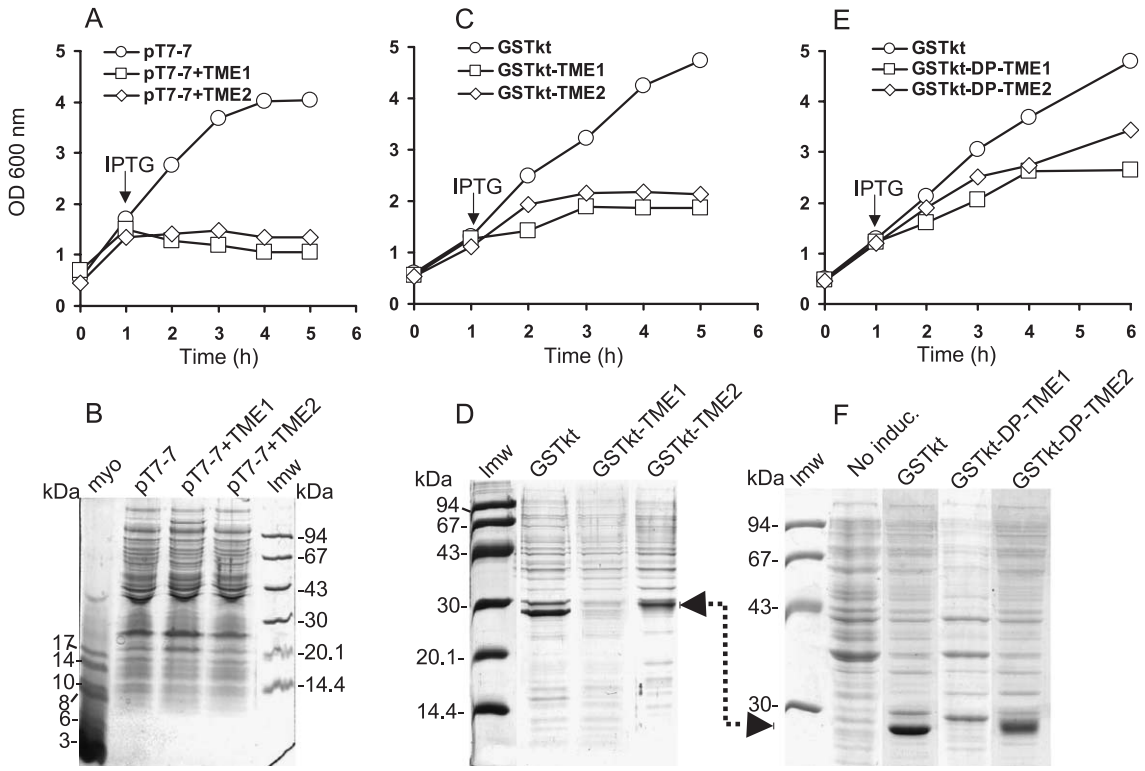


Fig. 2. Expression of TME1 and TME2 alone or in fusion with GSTkt in the presence or absence of the Asp-Pro sequence at the fusion junction. (Panels A, C, E) Growth curves of *E. coli* BL21(DE3)[pLysS] strain transformed with pT7-7 (A) or pGEXKT (C, E) expression vectors, either alone (circles) or coding for TME1 (squares) or TME2 (diamonds). When indicated, the Asp-Pro sequence (DP) was present at the junction between GSTkt and TME1 or TME2 (E). Clones were grown at 37 °C in liquid medium up to saturation. After dilution to a density of about 0.5 OD_{600nm}/ml in the same medium, expression was triggered after 1 h by the addition of IPTG as indicated. Cell density was followed as a function of time. (Panels B, D, F) SDS-PAGE analysis of bacterial proteins expressed after a 3-h induction with the different constructs. (B) Proteins expressed with the pT7-7 series; SDS-PAGE using a 16.5% polyacrylamide tricine gel (see Section 2). Lanes "myo" and "lmw" correspond to peptides from the Sigma "myo" kit and protein low weight markers from Amersham Biosciences, respectively. The corresponding molecular weights are indicated on the left and the right, respectively. (D and F) Proteins expressed in the pGEXTKT series with (F) or without (D) the Asp-Pro sequence inserted. SDS-PAGE using a 14% polyacrylamide Laemmli gel (see Section 2). GSTkt chimeras are indicated by arrows. The gel displayed in (F) was run over a longer period to improve the separation of the GSTkt chimeras.

Surprisingly, after transformation of the BL21(DE3)[pLysS] strain with the GSTkt-DP-TME2 chimera, expression was much less toxic for the host than that of the GSTkt-TME2 chimera without the Asp-Pro sequence (compare open diamond growth curves in Fig. 2E and C, respectively). Furthermore, this reduced toxicity was related to an increase in the production of the GSTkt-DP-TME2 chimera (Fig. 2F) when compared to GSTkt-TME2 production (Fig. 2D).

Introduction of the Asp-Pro sequence at the junction between GSTkt and TME1 (see Fig. 1) produced a similar effect. Expression of the GSTkt-DP-TME1 chimera was less toxic for the bacteria than GSTkt-TME1 (compare open square growth curves in Fig. 2E and C, respectively). Of particular interest is the fact that all the clones tested expressed the GSTkt-DP-TME1 chimera (Fig. 2E), while essentially no expression was detected for GSTkt-TME1 (Fig. 2D). As expected, both GST-DP-TM chimeras could be proteolyzed with acid formic, even when expressed as inclusion bodies (data not shown).

3.5. Insertion of the Asp-Pro sequence upstream of TME1 or TME2 expressed alone decreases the toxicity of TM domains

As insertion of the Asp-Pro sequence reduced TME1 and TME2 toxicity expressed in GSTkt chimeras, we tested whether this could also be efficient when inserted upstream of the TM domains expressed alone. The corresponding plasmids were generated from the pGEXKT constructs (as detailed in Section 2 and shown in Fig. 1) and BL21(DE3)[pLysS] competent strains were transformed and checked for growth before and after IPTG induction (Fig. 3). The resulting strains remained sensitive to the expression of either DP-TME1 (panel A, open squares) or DP-TME2 (panel B, open diamonds) upon IPTG induction, since they grew slower than the strain transformed with the empty plasmid (open circles). These strains appeared thus less sensitive to TME1 or TME2 toxicity when compared to those transformed with the plasmids expressing these TM domains alone (closed symbols). However, whatever the temperature used, ex-

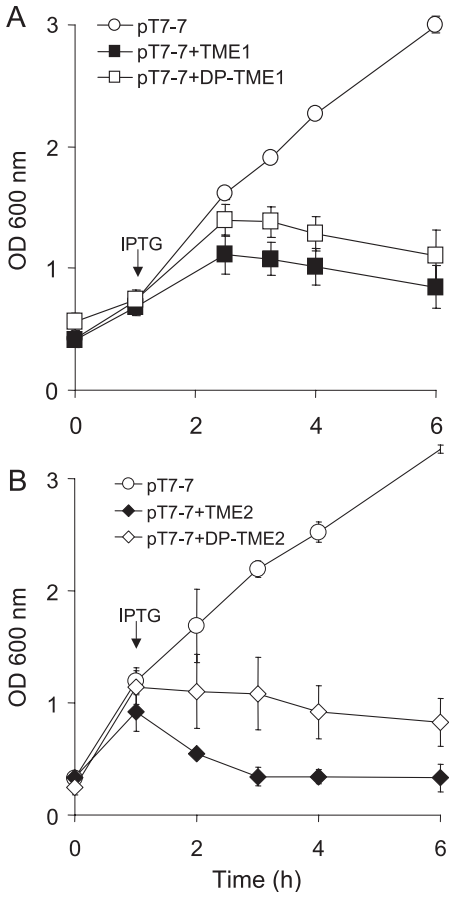


Fig. 3. Toxicity reduction of TM domain expression by insertion of the Asp-Pro sequence into pT7-7 vectors expressing TME1 or TME2 alone. Growth curves of *E. coli* strain BL21(DE3)[pLysS] transformed either with the empty vector pT7-7 or coding for TME1 or TME2 peptides, cloned alone or preceded by a DP coding sequence. Growth conditions, IPTG induction, and recording were as described in the legend to Fig. 2. Error bars were calculated from the data of three different experiments for each construct.

pression of DP-TME1 and DP-TME2 remained low since they could not be detected on a Coomassie blue-stained SDS-PAGE (not shown). Nevertheless, these data demonstrate that the addition of an Asp-Pro sequence alone contributes to lowering the toxicity induced by membrane domain expression.

3.6. Cellular localization of expressed GSTkt-DP-TME1 and GSTkt-DP-TME2

To better understand the reduction in TME1 and TME2 associated toxicity when expressed in fusion with the GST-DP sequence, we explored the cellular localization of the GSTkt-DP-TME1 and GSTkt-DP-TME2 chimeras. When protein expression was carried out at 37 °C, the chimeras accumulated mainly in the form of inclusion bodies (data not shown). These conditions are useful to produce large amount of chimeras but did not permitted detailed localization of chimeras in the cell by immuno-electron microscopy (see below). To reduce the level of inclusion bodies, the temperature of expression was reduced to 20 °C and induction time increased to 15–17 h. In addition, these conditions are commonly used to promote the folding of expressed proteins [9]. After expression, bacteria

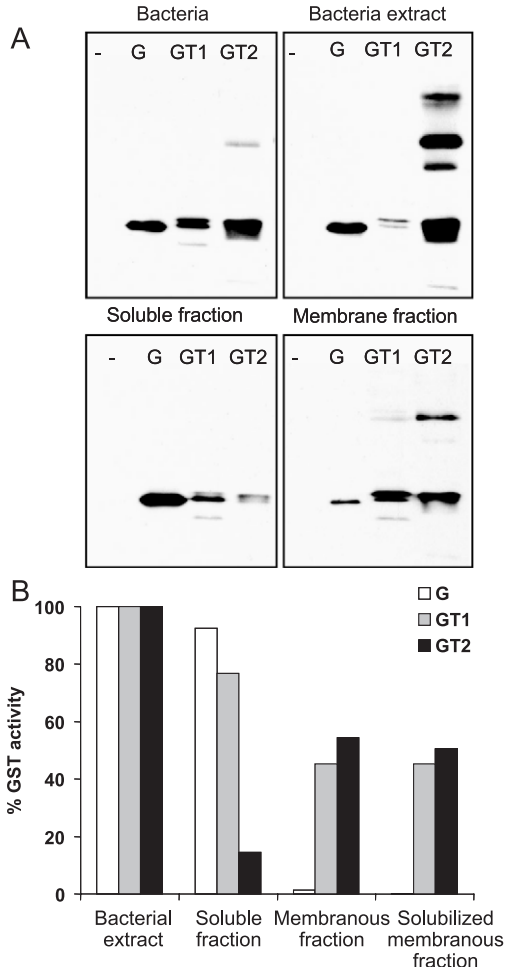


Fig. 4. Subcellular localization and GST activity of GSTkt-DP-TME1 and GSTkt-DP-TME2 chimeras by cellular fractionation. (A) Immunodetection of GST in various subcellular fractions of bacteria expressing GSTkt (G), GSTkt-DP-TME1 (GT1), and GSTkt-DP-TME2 (GT2) chimeras. Bacteria were grown at 37 °C and then cooled to 20 °C before induction and overnight expression at 20 °C, as described under Section 2. Subcellular fractionation was performed as detailed under Section 2. Bacteria were suspended, sonicated, and then submitted to low-speed centrifugation to yield the "bacterial extract" fraction. This fraction was submitted to high-speed centrifugation to give the "soluble fraction" and the "membranous fraction". For each fraction, protein were separated on a 12% SDS-PAGE and blotted onto Immobilon-P membranes before immunodetection of the GST moiety. (B) Activity of GST in the various subcellular fractions. The "solubilized membranous fraction" was obtained by solubilization of the "membranous fraction" with DM and then centrifuged to discard the non-solubilizable fraction. GST activity was measured as detailed under Section 2. GST activity of each expressed chimera is indicated as the percent of the activity measured in the "bacterial extract" fraction, which was fixed at 100%. Depending of the fraction and the chimera, the summation of the soluble and membranous fractions does not exactly correspond to 100%, probably due to a partial activation or inhibition of the GST in the presence of detergent.

were lysed by sonication and their components crudely separated by differential centrifugation followed by analysis by SDS-PAGE and Western blotting, using an antibody raised against the GST moiety (Fig. 4A). As illustrated in the "bacteria" panel, the GSTkt-DP-TME2 chimera was produced at about the same level as GSTkt alone. For unknown reasons, the band obtained with this chimera was always "fuzzy", as observed also in the Coomassie blue-stained gel (see Fig. 2F). This is possibly related to the presence of two cysteine residues in TME2. Indeed, mutations of Cys726 and Cys728 to Ala yielded a well-focused band for the corresponding GST-DP-TME2 mutant (data not shown). The GSTkt-DP-TME1 chimera could also be produced in reasonable amounts but two bands of about the same intensity were always observed in the "bacteria" fraction (Fig. 4A), despite the presence of PMSF and EDTA during sonication. The band of higher molecular weight is expected to be full-length GST-DP-TME1 while the lower band migrating at about the level of GST is likely an abortive chimera or a proteolytic product containing only a part of TME1.

Initial low-speed centrifugation released cell debris and inclusion bodies and about 2/3 of the proteins were recovered in the supernatant named "bacterial extract" (Fig. 4A). Both GSTkt and GSTkt-DP-TME2 chimeras were largely recovered in this fraction. In contrast, GSTkt-DP-TME1 could only be partially recovered, indicating that a large part of this chimera was still produced in the form of inclusion bodies. As illustrated in the "bacterial extract" and "bacteria" panels, the GSTkt-DP-TME2 chimera exhibited a tendency to form oligomers. This is probably partially due to the oxidation of cysteine residues present in TME2 despite the presence of β-mercaptoethanol during the fractionation process and SDS-PAGE analysis. Indeed, the amount of GSTkt-DP-TME2 oligomer was significantly lowered when the Cys residues were mutated to Ala (data not shown).

High-speed centrifugation of the "bacterial extract" fraction allowed the separation of "soluble" proteins from the "membranous fraction". As shown in Fig. 4A, the GSTkt chimera was found mainly in the "soluble fraction" while both GSTkt-DP-TME1 and GSTkt-DP-TME2 chimeras sedimented mainly with proteins of the "membranous fraction". Some oligomeric forms of both GSTkt-DP-TME1 and GSTkt-DP-TME2 could be observed in the latter fraction.

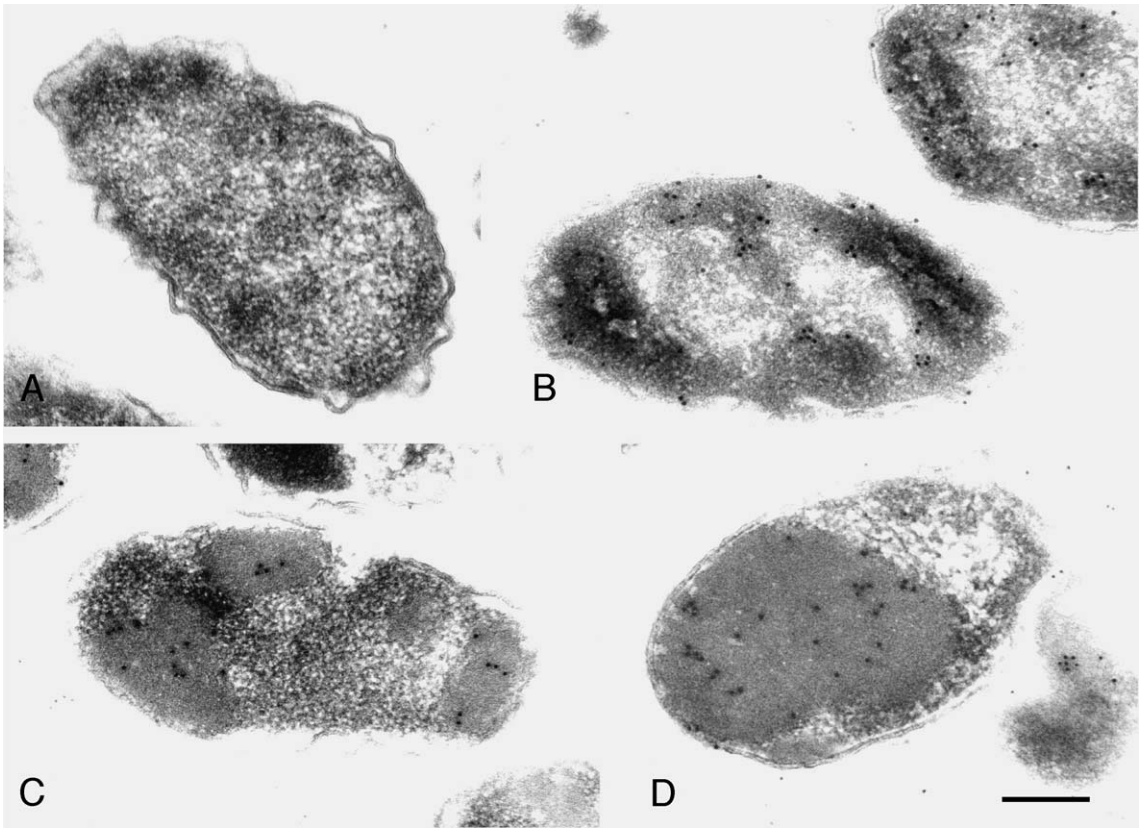


Fig. 5. Subcellular localization of GSTkt-DP-TME1 and GSTkt-DP-TME2 chimeras by immuno-electron microscopy. Bacteria were induced as described in the legend to Fig. 4 and then processed for immunolabeling as described under Section 2. After fixing and embedding the bacterial pellets in LR white resin, ultrathin sections were successively exposed to rabbit anti-GST polyclonal antibody and 10-nm gold particles conjugated with anti-rabbit IgG. (A) Control strain; (B, C, and D) bacterial strains expressing GSTkt, GSTkt-DP-TME1 and GSTkt-DP-TME2, respectively. Bar = 200 nm.

We further tested GST activity with respect to its capacity to transfer glutathione to 1-chloro-2,4-dinitrobenzene as described in Section 2. As shown in Fig. 4B, glutathione transferase activity was detected in the "bacterial extract" as well as in the "membranous fraction" containing GSTkt-DP-TME1 or GSTkt-DP-TME2 chimeras, indicating that the GST moiety in each chimera was functional, and thus well folded, despite its fusion with a hydrophobic membrane peptide. As expected for a soluble protein, GST activity associated with GSTkt was essentially found in the "soluble fraction" and not in the "membranous fraction". In contrast, some GST activity was found for GSTkt-DP-TME1 and GSTkt-DP-TME2 chimeras in the "soluble fraction", despite the presence of hydrophobic membrane peptide moiety. In the case of GSTkt-DP-TME1, the most abundant protein corresponds to the low molecular weight band supposed to be an abortive or proteolytic product of GST-DP-TME1 (see above). This product is likely responsible of the rather high GST activity observed in the corresponding "soluble fraction". In contrast, very limited amounts of abortive or proteolytic product are observed for GSTkt-DP-TME2 in the corresponding fractions. However, the presence of limited amount of full-length GSTkt-DP-TME1 and GSTkt-DP-TME2 chimeras in the "soluble fraction" suggests the existence of some soluble "micellar-like aggregates" small enough to remain in solution after centrifugation (see Section 4 below). Interestingly, solubilization of the "membranous fraction" by the mild detergent DM, followed by high-speed centrifugation, recovered all the GST activity in the "solubilized fraction" for both GSTkt-DP-TME1 and GSTkt-DP-TME2 chimeras (Fig. 4B). These results strongly suggest the presence of lipids associated with GSTkt-DP-TME1 and GSTkt-DP-TME2 chimeras since mild detergents as DM are not efficient to solubilize aggregated proteins or inclusion bodies. This constitutes a clear indication that the GST moiety is associated via TME1 or TME2 to some membranous structure. Moreover, the GST moiety of both GSTkt-DP-TME1 and GSTkt-DP-TME2, solubilized by DM and purified by affinity on GSH-agarose, was efficiently cleaved by thrombin (see Supplementary Material). This is in keeping with the native state of GST in both chimeras and constitutes a direct proof of the thrombin site accessibility provided by the design of our constructs.

3.7. GSTkt-DP-TME1 or GSTkt-DP-TME2 chimeras are clustered in the cytosol of E. coli

The above fractionation experiments suggest that the GSTkt-DP-TME1 and GSTkt-DP-TME2 chimeras could be associated with the bacterial membrane. To determine the subcellular localization of these chimeras, immuno-electron microscopy of sections of bacteria expressing the chimeras was carried out. Fig. 5 shows the immuno-gold electron microscopic detection of the GST moiety of GSTkt-DP-TME1 and GSTkt-DP-TME2 chimeras in the corresponding strain (panels C and D, respectively) compared to a strain expressing GSTkt alone (panel B) and a control strain (panel A). In all cases, the GST chimeras were detected in electron-dense regions with a homogeneous texture corresponding to the cytosolic compartment, but only exceptionally detected in or close to the membrane region, as in GSTkt (panel B). In addition, the gold particles were often found stacked together, forming clusters of varying size. All these data indicate that GSTkt-DP-TME1 and GSTkt-DP-TME2 chimeras are not associated with the bacterial membrane. Moreover, the presence of GST clusters related to the presence of lipids associated with GSTkt-DP-TME1 and GSTkt-DP-TME2 leads us to suppose that these chimeras form "proteo-lipidic aggregates" as tentatively represented in Fig. 6.

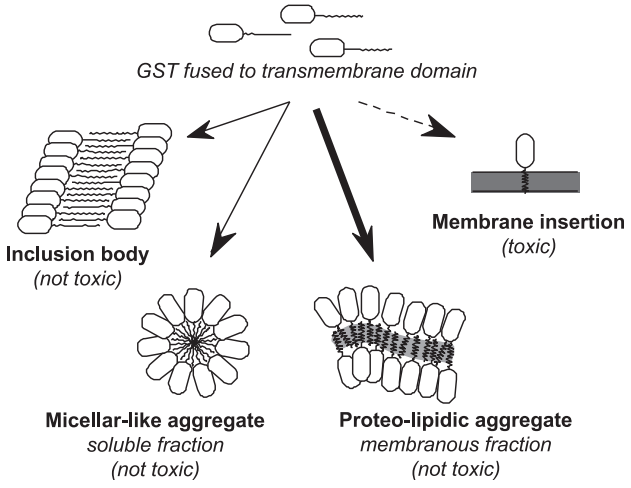


Fig. 6. Possible behavior of overexpressed TM domains fused to GST in bacteria. As the hydrophobic TM peptide is fused at the C-terminus of GST (symbolized as a globule), it is thought to be synthesized unfolded in the cytosol (symbolized as an extended chain). GST-TM chimeras could associate together by their hydrophobic TM peptides to form inclusion bodies (left, thin arrow). However, the presence of full-length GST-TM chimeras in the "soluble fraction" suggests the presence of some "micellar-like aggregate" (middle left, thin arrow) similar to the so-called "soluble inclusion bodies" described by Nomine et al. [45]. In such types of aggregate, the hydrophobic TM peptides are probably buried whereas the GST moieties are exposed to the cytosol. It is possible that some lipids associated with TM peptides could be present in both inclusion bodies and micellar-like aggregates. In such case, these lipids could allow the folding of TM peptides in α-helices. Because of its hydrophobic nature, the TM peptide could drive the binding and insertion of chimera to the bacterial membrane (right, dotted arrow), and fold into an alpha helix (symbolized as a helix). This pathway is probably toxic for the bacteria, due to membrane destabilization. Alternatively, the TM peptide could form "proteo-lipidic aggregates" (middle right, thick closed arrow) by association with lipids (indicated in gray) (see text for further explanation). Nevertheless and whatever the formation pathway, the various aggregates probably neutralize the toxicity of TM peptides by keeping them away from the bacterial membranes. Hence, any means of avoiding the targeting of hydrophobic TM peptides to bacterial membrane should result in reducing their toxicity. This could be achieved by the addition of an Asp-Pro sequence upstream of the TM sequences (see main text).

4. Discussion

The present work highlights an original property of the Asp-Pro sequence which contributes to a reduction in the strong toxicity associated with the expression of HCV E1 and E2 TM domains in bacteria and which promotes the production of GST-TM chimeras. This toxicity is thought to be due to the integration of TME1 and TME2 into the membrane, causing sufficient structural and/or functional disorders to kill the bacteria. The presence of an Asp-Pro sequence upstream of TME1 or TME2 is not sufficient by itself to promote the expression of these domains in appreciable amounts. Moving from the pT7-7 plasmid to the pGEXKT plasmid actually contributed to some reduction of the TM domains toxicity. This effect can be due to the presence of GST encoded by pGEXKT plasmid but also to the different promotion systems of respective plasmids. Indeed, it is known that T7 and *tac* promoters, present in pT7-7 and pGEXKT, respectively, can be critical for expressing membrane proteins in bacteria [9]. The Asp-Pro effect is more clearly evident when the dipeptide is inserted between the GST and TME1 or TME2 fusion proteins. Indeed, high levels of the corresponding chimeras (GSTkt-DP-TME1 and GSTkt-DP-TME2) could be produced in *E. coli*, while no or low expression was observed for GSTkt-TME1 and GSTkt-TME2 chimeras, respectively.

To better understand the reduced toxicity of TME1 and TME2 towards bacteria when expressed fused to a GST-DP sequence, we explored the cellular localization of the chimera by subcellular fractionation and immuno-gold electron microscopy. These experiments allowed us to propose a general scheme for the behavior of expressed TM domains when fused to GST in bacteria (Fig. 6). According to this scheme, when released from the ribosome, GST chimeras mainly aggregate in the cytosol in the form of inclusion bodies probably due to the stacking of their C-terminal membrane domains (Fig. 6, left). The presence of some full-length GSTkt-DP-TME1 and GSTkt-DP-TME2 chimeras in the soluble fraction suggests that parts of the chimeras could be organized as ‘‘micellar-like aggregates’’ (Fig. 6, middle left). These soluble aggregates are thought to be similar to the so-called ‘‘soluble inclusion bodies’’ that have been described by Nomine et al. [45] for the HPV E6 oncoprotein fused to maltose-binding protein. It is not clear whether these inclusion bodies and/or micellar-like aggregates contain lipids. In addition, the folding status of the TM peptides in these aggregates is not known. It is clear, however, that the retention of the TM peptides into these aggregates yields complexes that are non-toxic to the bacteria, though the formation of these aggregates does not explain the presence of active GST chimeras associated with lipids in the membranous subcellular fraction. Because of their hydrophobic nature, TM sequences located at the C-terminus of GST chimeras could behave as membrane insertion sequences and anchor the chimeras in the bacterial membrane, as observed for the so-called tail-anchored membrane proteins [46]. However, this pathway is likely to be toxic to the bacteria due to membrane destabilization. Furthermore, this pathway is not supported by our immuno-electron microscopy results since the GST chimeras were not detected in the membrane region of the bacteria but in the cytosolic compartment where they seem to form clusters. Taken together, these results suggest that the GST-TM chimeras probably form ‘‘proteo-lipidic aggregates’’ by association with cellular lipids. The structure of these aggregates is tentatively represented in Fig. 6 (middle right). Such aggregates could exhibit a similar density to that of the *E. coli* membrane, thus explaining their presence in the ‘‘membranous fraction’’. A key feature of these aggregates that distinguishes them from inclusion bodies is the presence of active GST in expressed chimera that can be solubilized with a mild detergent. Moreover, the GST moiety of both GSTkt-DP-TME1 and GSTkt-DP-TME2 was efficiently cleaved by thrombin. This finding opens new perspectives for the preparation of membrane peptides using such chimeras. This is also in keeping with the proposal that both TM peptides associated to the ‘‘proteo-lipidic aggregates’’ are in a ‘‘native’’ state. Indeed, the presence of lipids likely permits the folding of TM peptides into their native state, i.e., α-helices, which offer the most thermodynamically favored conformation in a membrane environment. Such folding is supported by our recent NMR structure analysis, which demonstrated that the N-terminal hydrophobic stretch of E1 forms an α-helix in a membrane environment [27]. Moreover, preliminary structural analyses of chemically synthesized peptides corresponding to TME1 and TME2 clearly indicate that both domains adopt an α-helical fold in any membrane mimetic environments tested (data not shown). These proteo-lipidic aggregates thus open interesting new perspectives for the structural and functional study of exogenous membrane domains expressed in bacteria. The formation and structure of these proteo-lipidic aggregates remain elusive but they are effective in neutralizing the potential toxicity of TM peptides by keeping them away from the bacterial membrane. However, one cannot exclude that GST-DP-TM chimeras initially insert into the membrane and then somehow bud off as proteo-lipidic aggregates.

The above results suggest that the addition of the Asp-Pro sequence upstream from TME1 or TME2 reduces their toxic effects by altering their binding to the bacterial membrane. This tendency is enhanced when a soluble protein such as GST is fused upstream of the construct. From the present work, it is not possible to have a clear-cut answer about the respective role of aspartic or proline residues or both for reducing the toxicity of TM peptides. However, the charge residue seems to have a critical importance. Indeed, Campion et al. [47] have shown that introducing negatively charges residues between phosphatase alkaline and human heregulin/human epidermal growth factor hybrids increased the level of expression of these

chimeras. In addition, TME2 that was always found less toxic than TME1 contains an acidic residue at its N-terminal end (Glu 717) while TME1 has a basic residue, His 352, at the same position. Although molecular mechanisms of peptide binding to the membrane remain poorly understood, it is generally observed that positively charged residues promote the binding of membrane peptide to the membrane through electrostatic interactions with negatively charged phospholipids [48,49]. On the contrary, negatively charged residues tend to decrease membrane binding due to electrostatic repulsion effect. Consequently, the additional negative charge born by the Asp-Pro sequence might prevent TME1 and TME2 peptides from binding to the membrane. The proline residue should have a more limited role since such a residue is already present at the C-terminal end of the GST while the corresponding chimeras remain toxic without DP. It is, however, not excluded that the particular structural features of the proline residue inserted with the Asp-Pro sequence favor the formation of stable chimera aggregates.

In conclusion, our results clearly indicate that the modification of the physico-chemical properties of the N-terminus of toxic membrane peptides could efficiently overcome their toxicity and permit their expression in bacteria. Remarkably, insertion of an Asp-Pro sequence upstream of a membrane peptide appears to be a useful method for preventing the toxicity of expressed membrane peptides by promoting their aggregation in the cytosol, and increasing their production. The properties of the Asp-Pro sequence could therefore be critical for successful expression of other toxic hydrophobic domains of membrane proteins in *E. coli* and should therefore contribute to increasing the number of structural and functional studies of these proteins.

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