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ORIGINAL RESEARCH PAPER

GroES and GroEL are essential chaperones for refolding of recombinant human phospholipid scramblase 1 in *E. coli*

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Abstract Human phospholipid scramblase 1(hPLSCR1), when expressed in *E. coli* (BL-21 DE3), forms inclusion bodies that are functionally inactive. We studied the effects of various stress inducing agents and chaperones on soluble expression of hPLSCR1 in *E. coli* (BL-21 DE3). Addition of 3% (v/v) ethanol before induction and decreasing the post-induction temperature to 15°C increased the solubility of hPLSCR1 to ~10 and ~15% respectively. Presence of *groES-groEL* complex solubilized the hPLSCR1 to ~30% of the total hPLSCR1. Absence of *groES-groEL* did not improve the solubility of hPLSCR1 suggesting that *groES* and *groEL* are the essential chaperones for the correct folding of hPLSCR1 when over-expressed in *E. coli*.

Keywords Chaperones · GroEL-GroES · Human phospholipid scramblase · Inclusion bodies · Membrane protein

Introduction

Mis-folding and aggregation of recombinant proteins in bacteria hinders the successful production of many eukaryotic proteins (De Marco et al. 2007). Phospholipid scramblases (PLSCRs) constitute a family of four homologous eukaryotic membrane proteins involved in Ca^{2+}-dependent, rapid, bi-directional movement (scrambling) of plasma membrane (PM) phospholipids (PL) in injured, apoptotic or activated cells (Basse et al. 1996; Sahu et al. 2007). Human phospholipid scramblase 1 (hPLSCR1) has been cloned and over-expressed in *E. coli* as maltose-binding fusion protein and purified using a maltose-binding column (Zhou et al. 2007). However, this process involves removal of the maltose-binding fusion protein to carry out further purification. Recently, we expressed $(His)_6$-hPLSCR1 in *E. coli* (BL-21DE3), purified it from inclusion bodies and refolded into native conformation by decreasing the concentration of denaturant (Sahu et al. 2008). However, the refolding yield is low (~5–10%). Therefore, it is essential to increase the yield of the protein in soluble form. Exposure of recombinant *E. coli* to heat shock (either by growing them at extreme temperature or in presence of ethanol) or osmotic shock (changing the concentration of osmolytes) is known to increase the solubility of recombinant proteins. Co-expressing the recombinant protein with chaperones is also exploited by various workers as the most effective way and a quality control system to increase the solubility of recombinant proteins in *E. coli* (Oganesyan et al. 2007; Weickert et al. 1997; Welch and Brown 1996; Young et al. 2004; Hartl and Martin 1995). In *E. coli*, folding of newly synthesized proteins is achieved either by KJE (dnaK with dnaJ and

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grpE) or ELS (groEL with groES) chaperone complexes. As most of these chaperones are heat-shock proteins that are over-expressed at extreme temperature or osmotic stress conditions (Buchberger et al. 1996; Jones and Inouye 2006), we investigated the effect of various stress inducing agents and co-expression of chaperones on the solubility of hPLSCR1.

Materials and methods

Materials

E. coli DH5α and *E. coli* BL-21 DE3 strains were obtained from ATCC. Plasmids containing chaperones were obtained from Takara Bio Inc. (Table 1), c-DNA of *hPLSCR1* was purchased from Invitrogen and *pET-28b*(+) was obtained from Novagen. IPTG, PMSF, DTT, EGTA, molecular biology grade $CaCl_2$ and other routine chemicals were purchased from Himedia (India). Egg phosphatidylcholine (egg PC) was obtained from Sigma and the fluorescent lipid, 7-nitrobenz-2-oxa-1,3-diazol-4-yl-phosphatidylcholine (NBD-PC), was purchased from Avanti Polar Lipids. Inc. SM2 biobeads and protein molecular weight markers were obtained from Biorad, (USA). The Ni^{2+}-NTA matrix used for the purification of hPLSCR1 was purchased from GE Health Care, USA.

Transformation and expression of chaperones and hPLSCR1 in *E. coli* (BL-21DE3)

The c-DNA of *hPLSCR1* flanked by *Eco* R1 and *Nde* I sites in *pET-28 b* (+) was transformed into *E. coli* BL-21 DE3 and grown in the selective media containing kanamycin (50 mg l^{-1}). The above cells when induced with 1 mM IPTG, produced protein hPLSCR1 with hexahistidine tag at its *N*-terminus (hereafter, the protein hPLSCR1 will mean (His_6)-hPLSCR1 if not specified otherwise). These cells were again transformed with different plasmids as shown in Table 1. The resulting strains formed are named as A (*hPLSCR1-pG-KJE8*), B (*hPLSCR1-pGro7*), C (*hPLSCR1-pKJE7*), D (*hPLSCR1-PG-Tf2*), and E (*hPLSCR1-PTf16*). All strains were able to grow in LB media containing kanamycin (50 mg l^{-1}) and chloramphenicol (20 mg l^{-1}). At OD_{600} ~0.3, tetracycline (5 μg ml^{-1}) and (or) arabinose (0.5 mg ml^{-1}) was added to the culture broth to over express the respective chaperones. The cells were again allowed to grow for ~2 h (OD_{600} ~0.6) and induced with 1 mM IPTG. The induction was carried out for 6 h at 37°C and 180 rpm. For optimization of the time of post induction, cells were collected at different post induction time (1–7 h) and harvested by centrifugation at 10,500×*g* at 4°C for 5 min.

Purification of the soluble $(His)_6$-hPLSCR1 by Ni^{2+}-NTA column and gel filtration

The cells were resuspended in 20 mM Tris–HCl, pH 7.5, 200 mM NaCl, 1 mM PMSF and 1 mM DTT. Lysis was carried out by a Vibrocell ultrasonicator for 2 min at 4°C with 1 s on and 2 s off cycles. The soluble and insoluble fractions were then separated by centrifugation at 10,500×*g*, 4°C for 20 min. The soluble fraction collected from induced cells after lysis and centrifugation was loaded on to 5.0 ml Ni^{2+}-NTA column equilibrated with 20 mM Tris/HCl, 200 mM NaCl, pH 7.5. The bound fraction was

Table 1 Plasmids containing various chaperones used to increase the solubility of hPLSCR1 when over expressed in *E. coli* and their corresponding solubility

No.	Plasmid	Chaperone	Promoter	Inducer	Resistant marker on the plasmid	% Solubility of $(His)_6$-hPLSCR1[a]
1	pET-28b(+)-*hPLSCR1*	No chaperone (control)	T_7	IPTG	Kanamycin	4 ± 1.1
2	pG-*KJE8*	*dnaK-dnaJ-grpE*	araB	L-Arabinose	Chloramphenicol	30 ± 2.4
		groES-groEL	Pzt1	Tetracycline		
3	PGro7	*groES-groEL*	araB	L-Arabinose	Chloramphenicol	20 ± 3.1
4	pKJE7	*dnaK-dnaJ-grpE*	araB	L-Arabinose	Chloramphenicol	5.5 ± 2.3
5	PG-Tf2	*groES-groEL-tig*	Pzt1	Tetracycline	Chloramphenicol	20 ± 1.8
6	PTf16	*tig*	araB	L-Arabinose	Chloramphenicol	6 ± 1.5

[a] The % solubility values reported are mean of three different experiments

successively washed with the equilibration buffer containing 25 mM and 50 mM imidazole (10 column volumes each). The bound $(His)_6$-hPLSCR1 was eluted by equilibration buffer containing 250 mM imidazole. The protein recovered in this process was almost pure. This fraction was then loaded to superdex-200 gel filtration column (the mobile phase was 20 mM Tris/HCl, 200 mM NaCl, pH 7.5, flow rate 1 ml/min) to get the hPLSCR1 in pure form.

Effect of ethanol on the solubility of hPLSCR1

The cells containing *hPLSCR1* in *pET-28 b* (+) were grown in the LB broth containing kanamycin (50 mg l^{-1}). At OD_{600}–0.4, different concentration (2–7% v/v) of ethanol were added to analyze the effect of its concentration on the solubility of hPLSCR1. The cells were again grown for 1 h (OD_{600} ~0.6) and expression of hPLSCR1 was induced by addition of 1 mM IPTG. The induction was carried out for 6 h at 37°C and 180 rpm. The cells were harvested as described earlier.

Effect of temperature on the solubility of hPLSCR1

The effect of temperature on the solubility of hPLSCR1 was determined by growing the cells up to OD_{600} ~0.6 and inducing the expression of hPLSCR1 by adding 1 mM IPTG. After induction, cells were allowed to grow at different temperatures (15°C, 25°C, 30°C, 37°C and 42°C) for 6 h and 180 rpm. The cells were harvested as described earlier.

Electrophoresis techniques and measurement of protein solubility

The insoluble fractions were dissolved by heating them in lysis buffer containing 1% SDS and heated at 100°C for 15 min. The expression of chaperones and hPLSCR1 in whole cell (W), insoluble (I) and soluble (S) fractions were checked on 12% SDS-PAGE. The gels were analyzed in a Biorad gel documentation system and amount of hPLSCR1 (~37 kDa) present in each fraction was measured by comparing their band intensity with a known concentration of hPLSCR1 that was purified from inclusion bodies.

Determination of non-aggregated state of the chaperone solubilized hPLSCR1

The purified hPLSCR1 was subjected to gel permeation chromatography through Superdex-200 column equilibrated with 20 mM Tris/HCl, 200 mM NaCl, pH 7.5 at 1 ml/min at 25°C. The total column volume was 120 ml and void volume (V_0) was 45.5 ml. Both the chaperone solubilized hPLSCR1 and urea solubilized inclusion bodies of hPLSCR1 were passed through the column to determine the non-aggregated state of hPLSCR1 (37 kDa). 1 mg of the inclusion body (dissolved in 20 mM Tris/HCl, 200 mM NaCl, pH 7.5. containing 6 M urea) as well as chaperone solubilized hPLSCR1 (in 20 mM Tris/HCl, 200 mM NaCl, pH 7.5) were passed through the column. The approximate molecular weight of the eluted hPLSCR1 was determined from the elution volumes (V_e) of the protein and the standard curve.

Reconstitution of the soluble hPLSCR1 into proteoliposomes

Symmetrically labeled vesicles with hPLSCR1 reconstituted into them (proteoliposomes of hPLSCR1) were prepared as described earlier (Chang et al. 2004; Gummadi and Menon 2002). Briefly, 4.5 μmol egg PC was mixed with 0.3 mol% NBD-PC was dried under N_2 to remove chloroform completely. The dried mixture was solubilized in 10 mM HEPES/NaOH, pH 7.5, 100 mM NaCl, 1% Triton X-100 along with 100 μg hPLSCR1 and detergent was removed using SM2 biobeads (0.3 g ml^{-1}). The proteoliposomes were collected by ultracentrifugation at 1,50,000×*g*. These symmetrically labeled vesicles were quenched on outside leaflet using 20.0 mM dithionite (irreversible quenching agent) to get inside labeled vesicles.

Assay for the scramblase activity

The most common way to measure the scramblase activity is to insert the protein into artificial membrane (e.g. liposomes) and to measure the translocation of a phospholipid analog that has been attached with a tracer group (e.g. NBD, that provides fluorescence to the protein). In the present study, we studied the translocation of NBD labeled phosphatidylcholine

(NBD-PC) to determine the scramblase activity of hPLSCR1. Liposomes (vesicles without any protein) and proteoliposomes (vesicles containing hPLSCR1) were prepared with ~97% of egg-PC and ~3% NBD-PC. 100 μg hPLSCR1 was taken for reconstitution of both soluble hPLSCR1 and Triton-X-100 dissolved inclusion bodies to compare their scramblase activities. NBD-PC was either equally distributed in both the leaflets of lipid vesicles (symmetrically labeled) or was made to be present in the inner leaflet (inside labeled). Scramblase activity was measured for both the inside as well as symmetrically labeled vesicles as described earlier with little modification (Basse et al. 1996; Sahu et al. 2008). Briefly, the liposomes and proteoliposomes were incubated for 2 h at 37°C in 10 mM HEPES/NaOH, pH 7.5, 100 mM NaCl in the presence of either 2 mM Ca^{2+} or 4 mM EGTA. The vesicles were then transferred into cuvette with continuous stirring at 25°C and initial fluorescence was recorded at excitation 470 nm and emission 530 nm for 100 s in a spectrofluorimeter. After the initial fluorescence was stabilized, 20 mM dithionite was added to quench the fluorescence present in the outer leaflet of liposomes and proteoliposomes. The difference between the non-quenchable fluorescence observed in the presence and absence of Ca^{2+} (presence of EGTA) was due to the Ca^{2+} induced scrambling of NBD-PC by hPLSCR1.

Results and discussion

Effect of ethanol on the solubility of hPLSCR1 expressed in *E. coli*

We used different concentrations of ethanol to study its effect on the solubility of hPLSCR1 in *E. coli* BL-21DE3. The cells were added with increasing concentration of ethanol to expose them to increasingly ethanol stressed condition. Solubility of hPLSCR1 increased from 4% to about 10% when expressed in the presence of 3% (v/v) ethanol (Fig. 1a). Highest solubility of hPLSCR1 was observed when 3% (v/v) ethanol was added to the culture media before inducing with IPTG. Ethanol has been used to increase the solubility of cytochrome C 450 (Kusano et al. 1999) and preS2-S′-β-galactosidase (Thomas and Baneyx 1997). Ethanol at 3% (v/v) increased the solubility of recombinant lipooxygenase by 40% (Steczko et al. 1991). Ethanol is a powerful elicitor of heat-shock response and induces the expression of many heat shock proteins including dnaK-dnaJ-grp-E and groES-groEL machineries in bacteria. Hence, the observed increase in solubility of hPLSCR1 upon addition of ethanol was probably due to an up regulation of heat shock chaperones in *E. coli*.

Effect of temperature on the solubility of hPLSCR1 expressed in *E. coli*

Effect of different post induction temperature on solubility of hPLSCR1 was studied on *E. coli* (BL-21DE3) strain expressing hPLSCR1. Cells, grown at post-induction at 15°C, expressed up to 15% hPLSCR1 in the soluble fraction (Fig. 1b). Below 15°C did not improve the protein yield due to poor growth of cells. Higher temperatures gave good yields in total cellular protein but decreased the solubility of hPLSCR1.

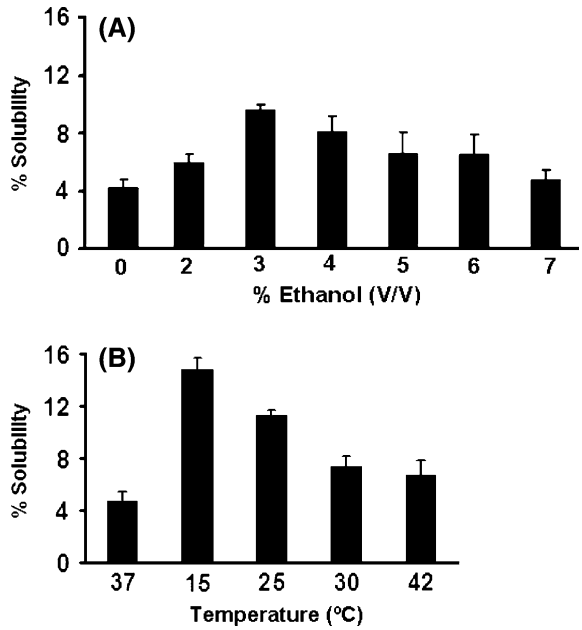

Fig. 1 Effect of stress inducing agents on the solubility of hPLSCR1. **a** Effect of ethanol on the solubility of hPLSCR1. **b** Effect of post induction temperature on the solubility of hPLSCR1. Intensity of the hPLSCR1 (37 kDa) was compared with a known concentration (5 mg/ml) of hPLSCR1 purified from inclusion bodies to calculate the amount of hPLSCR1 present in each lane in SDS-PAGE. The figure shows the representative result of three independent sets of experiments with ±1 to ±10% standard deviation

At 37°C, the expression of hPLSCR1 was ~4% whereas 42°C gave a slight increase in the solubility of hPLSCR1. A decrease in temperature increases the activity of chaperones (Sørensen and Mortensen 2005; Ferrer et al. 2003). The cold-shock proteins, such as CspA and RbfA, are induced at low temperature and are associated with a number of essential cellular activities such as transcription, translation, m-RNA degradation and recombination (Jones and Inouye 2006; Jones et al. 1987; Ferrer et al. 2003). Our study shows that low temperature conditions enhances the synthesis of chaperones required for folding of hPLSCR1 into its native conformation.

Effect of chaperones on the solubility of hPLSCR1

We used different combination of chaperones to enhance the solubility of hPLSCR1 in *E. coli* BL21DE3 (Table 1). The solubility was found to be ~20% of the total hPLSCR1 when expressed in presence of *pGro7* and *PG-Tf2*. However, no enhancement in solubility (compared to control) was observed when hPLSCR1 was co-expressed with *pKJE7I* or *PTf16* (Table 1). Maximum solubility (30% of total hPLSCR1) was achieved 6 h post-induction of hPLSCR1 when co-expressed with *pG-KJE8* (Table 1, Fig. 2a). The soluble fraction was partially purified in Ni^{2+}-NTA column and was further purified by gel-permeation chromatography (Fig. 2b). hPLSCR1 purified from inclusion bodies was used as a control to determine the non-aggregated state of the soluble hPLSCR1. The urea solubilized hPLSCR1 showed aggregation to a very high molecular weight (>200 kDa) (Fig. 2c trace a), whereas soluble hPLSCR1 showed monomeric state (~37 kDa) as shown by the elution volume of hPLSCR1 (Fig. 2c trace b). In eukaryotic cells, folding of proteins is catalyzed by a defined set of molecular chaperones. However, key chaperone(s) required for correct folding varies for different proteins. As the chaperones are normally expressed at a low level in prokaryotic cells, overexpression of eukaryotic proteins with chaperones has been used by various workers to enhance the solubility of the over expressed protein in *E. coli* (Welch and Brown 1996). Different proteins have been co-expressed with different combinations of chaperones to enable their optimum expression in soluble fraction (Welch and Brown 1996; Schröder et al. 1993; De Marco et al. 2007). Our study shows that the presence of chaperone complexes those contain GroES and GroEL (e.g. plasmids *pG-KJE8, pGro7* and *PG-Tf2*) increased the solubility of hPLSCR1. However, plasmids *pKJE7I* and *PTf16* that did not contain GroES or GroEL did not increase the solubility of hPLSCR1. These findings suggest that GroES and GroEL are essential for correct folding of hPLSCR1 when over expressed in *E. coli*.

Solubilized hPLSCR1 shows phospholipid scrambling activity

To check for the biological activity of recombinant hPLSCR1 purified from the soluble fraction we performed scramblase assay (as described in materials and methods) with both symmetrically as well as inside labeled vesicles. The inside labeled vesicles incubated with 4 mM EGTA (absence of Ca^{2+}) showed no decrease in initial fluorescence intensity upon addition of dithionite, where as a slow decrease of fluorescence intensity was observed for vesicles incubated with 2 mM $CaCl_2$ (Fig. 3b). The decrease in fluorescence intensity of vesicles incubated with 2 mM $CaCl_2$ was due to the quenching of NBD-PC molecules that have been translocated to the outer leaflet from the inner leaflet by the activated hPLSCR1. Our results show that the liposomes (control vesicles) those are devoid of any protein in them do not show scrambling activity (Fig. 3a). Scramblase assay was also performed for symmetrically labelled vesicles as another control. In the presence of EGTA, hPLSCR1 gets inactivated and NBD-PC on the outer leaflet (~50%) gets quenched by dithionite, thus showing 50% decrease in fluorescence intensity (Fig. 3b). Under active conditions of hPLSCR1 (symmetrically labelled vesicles incubated with Ca^{2+}), shows no movement of probe (50% quenching in NBD fluorescence similar to that obtained in the presence of EGTA), which is consistent with bidirectional phospholipid exchange between the two leaflets (Fig. 3b) (Basse et al. 1996).

However, when inclusion bodies were dissolved in 1% Triton-X-100 and reconstituted into proteoliposomes, the protein did not show any scramblase activity, indicating that aggregated hPLSCR1 in the

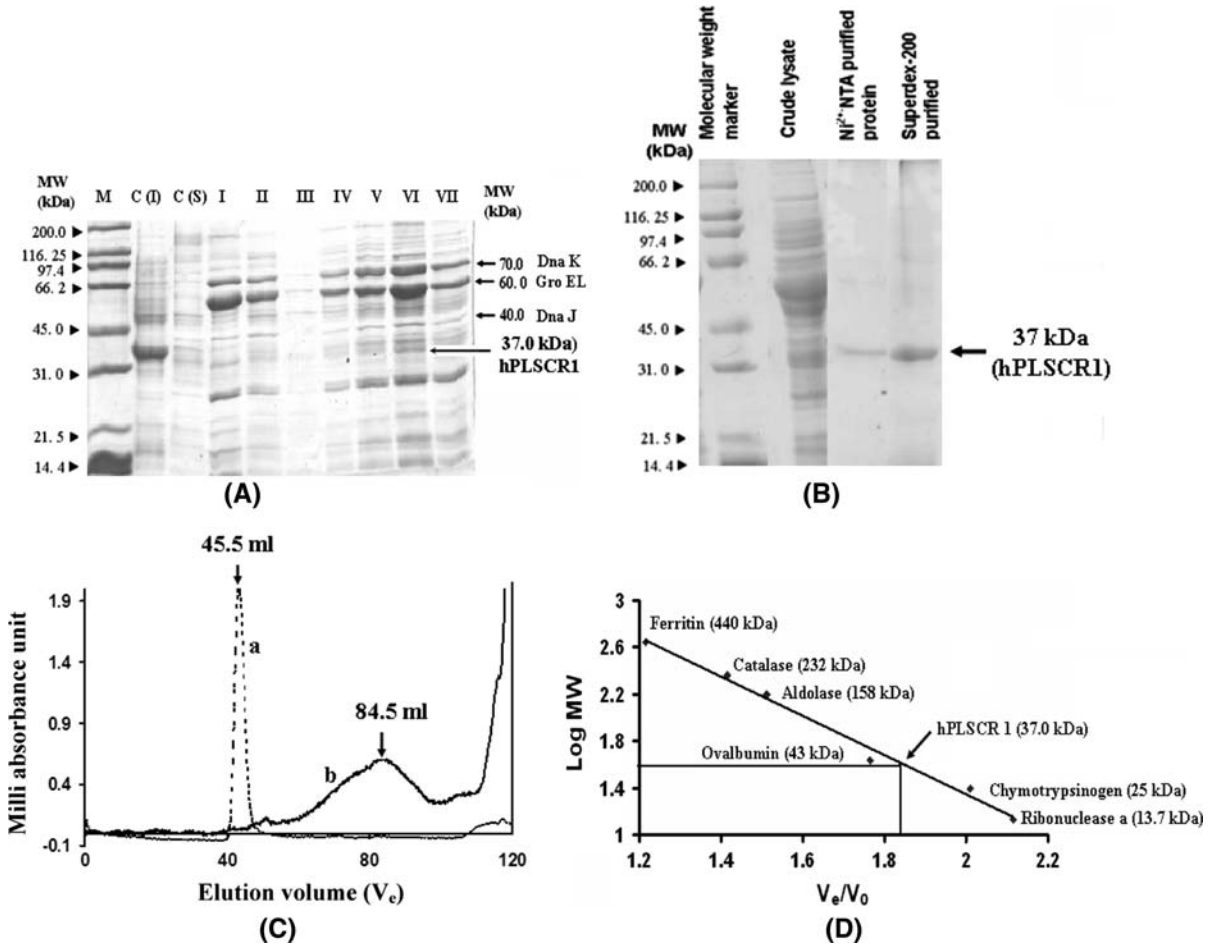

Fig. 2 Over-expression, purification and non-aggregated state of soluble hPLSCR1. **a** SDS-PAGE showing the effect of co-expression of *pG-KJE8* (containing chaperones dnaK–dnaJ–grpE, gro-ES-groEL) on soluble expression of hPLSCR1. C(I) and C(S) represent the insoluble and soluble fractions of control (chaperone free) cells. I–VII represent the soluble fractions from 2 to 7 h of post induction samples of *E. coli* (BL-21DE3) cells expressing chaperones and hPLSCR1. The amount of protein loaded in each lane was normalized according to OD_{600}. **b**. Purification of hPLSCR1 from the soluble fraction by Ni^{2+}-NTA column and gelfiltration. **c** Gel filtration chromatography showing the non-aggregated state of hPLSCR1. Urea dissolved inclusion bodies show aggregation to a higher order molecular weight (>200 kDa) that does not enter the column as shown by its elution volume (Ve $\sim$ 45.5 ml, $V_e/V_0 \sim 1$) (trace a), Chaperone-solubilized hPLSCR1 ($V_e = 84.5$ ml, $V_e/V_0 \sim 1.83$) corresponds to the monomeric state of the protein ($\sim$37 kDa) (trace b). **d** Standard curve showing the linear relationship between the V_e/V_0 of the proteins of known molecular weight to their log(MW). Elution volume of soluble hPLSCR1 corresponds to $\sim$37 kDa, i.e. monomeric state of the protein

insoluble fraction is biologically inactive (Fig. 3c). About 10% of labelled phospholipid was translocated in 2 h (Fig. 3b) and this rate of translocation was comparable to previously published reports (Sahu et al. 2008, Basse et al. 1996). However, these translocation rates are lower that obtained from resealed erythrocyte and platelet membranes. This discrepancy between the in vivo and in vitro scramblase activities can either be explained by considering the presence of accessory factors present in the intact membranes that is essential for the enhanced scramblase activity in vivo or our inability to incorporate the desired quantity of hPLSCR1 protein in the proteoliposomes (Zhou et al. 1997; Sahu et al. 2007).

In summary, we showed increase in solubility of a human plasma membrane protein hPLSCR1 by using stress inducing agents ethanol, subjecting the

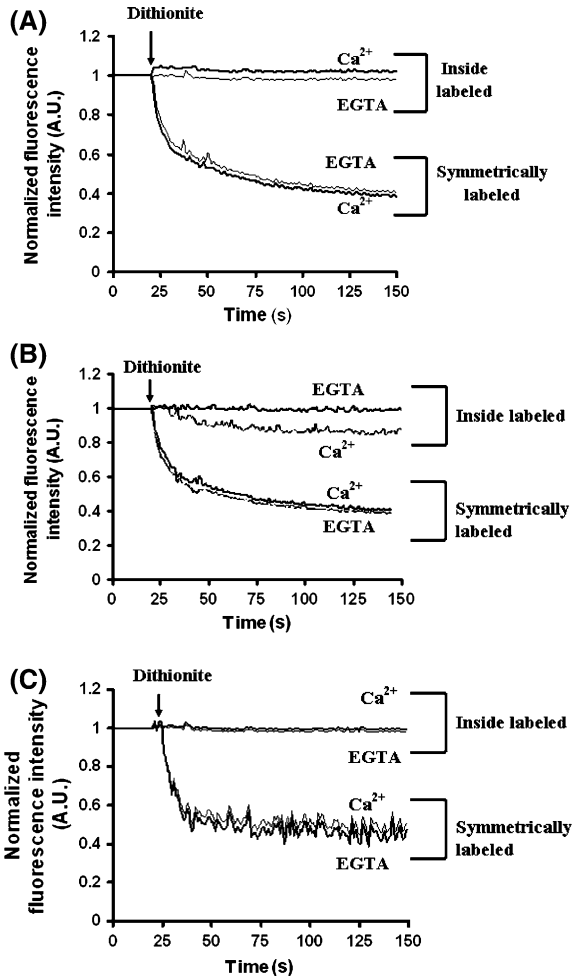

Fig. 3 Scrambling activity of soluble hPLSCR1. **a** Control (liposomes), **b** Soluble fraction of hPLSCR1 co-expressed with *pG-KJE8* (chaperones dna K-dna J-grpE, gro-ES-groEL), **c** Inclusion bodies of hPLSCR1 dissolved in 1% TritonX-100. Same amount of hPLSCR1 (100 μg) was taken for reconstitution in Fig. 3a and b. The assay was performed in 10 mM HEPES/NaOH; pH 7.5 containing 100 mM NaCl at 25°C in a JASCO-6500 spectrophotometer. The figure shows the representative result of three independent sets of experiments with ±1 to ±5% standard deviation

cells to cold shock and co-expressing the protein it with chaperones GroES and GroEL. Our study shows that GroES and GroEL are essential chaperones for folding of hPLSCR1 in *E. coli*.

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