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Membrane binding of human phospholipid scramblase 1 cytoplasmic domain

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ABSTRACT

Human phospholipid scramblase 1 (SCR) consists of a large cytoplasmic domain and a small presumed transmembrane domain near the C-terminal end of the protein. Previous studies with the SCRΔ mutant lacking the C-terminal portion (last 28 aa) revealed the importance of this C-terminal moiety for protein function and calcium-binding affinity. The present contribution is intended to elucidate the effect of the transmembrane domain suppression on SCRΔ binding to model membranes (lipid monolayers and bilayers) and on SCRΔ reconstitution in proteoliposomes. In all cases the protein cytoplasmic domain showed a great affinity for lipid membranes, and behaved in most aspects as an intrinsic membrane protein. Assays have been performed in the presence of phosphatidylserine, presumably important for the SCR cytoplasmic domain to be electrostatically anchored to the plasma membrane inner surface. The fusion protein maltose binding protein-SCR has also been studied as an intermediate case of a molecule that can insert into the bilayer hydrophobic core, yet it is stable in detergent-free buffers. Although the intracellular location of SCR has been the object of debate, the present data support the view of SCR as an integral membrane protein, in which not only the transmembrane domain but also the cytoplasmic moiety play a role in membrane docking of the protein.

1. Introduction

Human phospholipid scramblase 1 (SCR) is an endofacial, calcium-dependent monotopic membrane protein, which is associated to lipid rafts when multipalmitoylated [1,2]. SCR is a member of a family of membrane proteins that has been proposed to catalyze the Ca^{2+}-dependent, ATP-independent transbilayer lipid motion, or flip-flop, thus leading to the loss of membrane lipid asymmetry [1,2]. SCR has a broadly globular structure with a hydrophobic stretch near the C-end (residues 291–309) that is presumed to act as a transmembrane domain (TMD) that would anchor the protein to the membrane. Previous work from this laboratory with SCRΔ, the human phospholipid scramblase 1 (SCR) mutant lacking the C-terminal 28 aa, supports the hypothesis of the presence in the protein of a TMD in the C-terminal end, as a determinant for the lipid "scrambling" and calcium binding activities [3], in agreement with the observations by Francis et al. [4]. The TMD is close to the calcium-binding domain in the protein sequence, and both could be mutually regulated. Calcium binds both the wild type and mutant proteins but the lack of TMD decreases the calcium-binding affinity of SCRΔ by about 5–10 fold and affects protein folding and stability. Peptides representing the presumed TMD have been shown to become inserted in lipid bilayers of different compositions [5].

It has been reported [6] that SCRΔ, when expressed in Jurkat cells, is no longer localized preferentially in the plasma membrane, but rather distributed evenly in the cytosol. However the precise intracellular location and even the function of SCR have been disputed. When SCR is palmitoylated it partitions with EGF receptor in lipid rafts [1]. In the absence of palmitoylation, virtually all of the expressed SCR localizes to the nucleus [2] where the protein binds a genomic DNA with high affinity suggesting a potential function as a transcription factor [7,8]. These data suggest that the post-translational acylation determines the protein localization in the cell and regulates its normal function, either in the nucleus or incorporated to the membrane [2,9].

A subsequent structural model computed by homology modeling suggests that the C-terminal transmembrane helix is buried within the SCR core and that palmitoylation may represent the principal membrane anchorage for the protein [10]. Other studies reveal that SCR is secreted via a lipid-raft dependent mechanism and deposited in the extracellular matrix, suggesting that SCR is a multifunctional protein that can function both inside and outside of the cell. In addition, the

Abbreviations: LUVs, large unilamellar vesicles; MBP, maltose binding protein; OG, octylglucoside; PC, phosphatidylcholine; PS, phosphatidylserine; SCR, human phospholipid scramblase 1; SCRΔ, human phospholipid scramblase 1 mutant [^{1}M-K^{290}]; TMD, transmembrane domain

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latter study suggests that palmitoylation is most likely not involved in the trafficking and secretion of SCR [11].

In our previous study [3] we found that SCRΔ in the presence of Ca^{2+} could not promote lipid translocation, at variance with the wild-type, thus suggesting that the TMD was important both for Ca^{2+} binding and for the protein overall active conformation. In the present study we intend to show that the truncated form in the absence of Ca^{2+} shares many similarities with the wild-type protein in its interaction with membranes. We have clarified the effect of TMD suppression on protein binding to model membranes made of egg PC or PC:PS (9:1 mol ratio) (the latter mimicking the cell membrane inner monolayer). We have also analyzed the reconstitution process behavior of SCRΔ. Demonstrating that the truncated SCRA can still bind to lipid bilayers is important because there is still some debate about the cell location and in vivo function of SCR [4,10,11]. Our SCRΔ preparation, expressed in *Escherichia coli*, is not palmitoylated thus it is less likely to interact with membranes than the corresponding domain expressed in eukaryotic cells. Even so the results demonstrate that the SCR cytoplasmic domain features flexible and adaptive interactions with the surrounding membrane, behaving in crucial aspects like an intrinsic membrane protein. A parallel study of the fusion protein maltose binding protein (MBP)-SCR describes an intermediate case of an artificial form of the protein that can bind the hydrophobic membrane core, yet it can remain stable in solution in the absence of detergents.

2. Materials and methods

2.1. Materials

Egg phosphatidylcholine (PC) and spinal cord phosphatidylserine (PS) were purchased from Avanti Polar Lipids (Alabaster, AL) and Lipid Products (South Nutfield, UK), respectively. The monoclonal anti-scramblase antibody was from Abcam (Cambridge, UK) and HRP-linked anti-mouse antibody was from New England Biolabs (MA, USA). D_2O was from Apollo scientific (Cheshire, UK). Octylglucoside was from Calbiochem (USA). Triton X-114 was from Sigma (MO, USA). All other reagents were of analytical grade.

2.2. Protein purification

SCRΔ purification was achieved by extracting the protein from inclusion bodies [3]. Briefly, SCRΔ was expressed in *E. coli* BL21-Codon Plus and protein over-expression was induced with 1 mM IPTG for 12 h at 16 °C. Cells were harvested by centrifugation and resuspended in "lysis buffer" (200 mM NaCl, 1 mM DTT, 1 mM EDTA, 20 mM Tris, pH 7.5) and treated with lysozyme. The samples were then sonicated, the suspensions centrifuged, and the inclusion bodies recovered in the pellet. After several washing steps, the pellets were resuspended in "TU buffer" (6 M urea, 50 mM NaCl, 0.1 mM TCEP, 20 mM Tris, pH 7.5) and centrifuged to collect the soluble fraction. The sample in TU buffer was then applied to a HisTrap HP column and the protein was eluted in a stepwise 0 to 500 mM imidazole gradient in the same TU buffer. Finally, the protein underwent overnight dialysis in order to remove the urea.

MBP–SCR was expressed in *E. coli* BL21 Codon plus strain. The fusion protein was first purified using an amylose resin and then diluted 5-fold with "T-A buffer" (1 mM DTT, 20 mM Tris, pH 7.5) and applied to a DEAE–Sepharose ion exchange column. Finally the protein was eluted in a stepwise 0 to 150 mM NaCl gradient in the same buffer. MBP–SCR was not subjected to cleavage with Factor Xa.

2.3. Surface pressure measurements

Lateral pressure experiments were carried out in a multi-well Delta Pi-4 Langmuir balance (Kibron Inc., Helsinki, Finland) under constant stirring. SCR- and SCRΔ-induced changes in surface pressure at the air–water interface and protein–lipid monolayer interactions were studied at 25 °C. Monolayers were formed by spreading a small amount of the lipid mixtures in chloroform:methanol (2:1 v/v) solution on top of assay buffer, until the desired initial surface pressure was reached. Proteins were injected with a micropipette through a hole connected to the subphase with constant stirring. The assay buffer was 50 mM NaCl, 20 mM Tris, pH 7.4.

2.4. SCR and SCRΔ binding to LUVs

PC:PS (9:1 mol:mol) LUVs, corresponding to 1 μmol lipid were diluted to 1 ml D_2O buffer (100 mM KCl, 0.1 mM EGTA, 20 mM Tris, pH 7.4) together with the appropriate amount of protein, to give a protein:lipid molar ratio of 1:1000. The mixture was allowed to equilibrate for 30 min at room temperature, and then centrifuged in a 120.2 Beckman rotor (500,000 ×*g*, 2 h, 20 °C) [12]. In this fashion the free, but not the lipid-bound protein will sediment when centrifuged. 100 μl were recovered and assayed for lipid and protein quantifications by phosphorous and dot blot, respectively. Briefly, dot blots for protein quantification were performed using a Hybond-C extra (Amersham Biosciences) membrane. The D_2O gradient-derived samples were spotted onto the membrane and blocked with 5% skim milk for 1 h at room temperature, followed by 1 h incubation at room temperature with anti-scramblase antibody (1:1000). The blot was washed several times with PBS, pH 7.4, and incubated for 1 h with an HRP-linked anti-mouse antibody (1:2000). After final washings to eliminate the unbound secondary antibody, the blot was developed on a Curix 60 processor (AGFA, Belgium) using Amersham Hyperfilm ECL (GE Healthcare, UK). The intensity of the sample signal was measured with a GS-800 densitometer (Bio-Rad, Stockholm, Sweden).

2.5. SCR and SCRΔ reconstitution analysis

Either SCRΔ or MBP–SCR proteins were incubated with LUVs (at a 1:800 protein-to-lipid mol ratio) in 100 mM KCl, 0.1 mM EGTA, 100 mM Tris, pH 7.5, in the presence of saturating concentrations of octyl-β-glucopyranoside (27–34 mM) for 1 h followed by overnight dialysis in the presence of SLM-Aminco BioBeads (2 g/l). The recovered samples were next dialyzed against D_2O buffer in Slide-A-Lyzer Mini Dialysis units. Samples were then ultracentrifuged in a TLA 120.2 Beckman rotor at 500,000 ×*g* for 2 h at 20 °C. The various gradient fractions were recovered in 100 μl aliquots. The polycarbonate centrifuge tubes were then washed with 100 μl hot 1% (w/v) SDS to recover protein that had been aggregated or adhered to the tube walls.

2.6. Solubilization by Triton X-114

The upper two fractions recovered from the isolated reconstituted D_2O proteoliposomes of MBP–SCR or mutant SCR were dissolved in 1% (w/v) Triton X-114 at 4 °C. Then, the sample was heated up to the detergent cloud point, at 30 °C for 10 min, and centrifuged at low speed (1000 ×*g*, 3 min, 30 °C) to facilitate phase separation [13–15]. Aliquots from the upper (detergent poor) and lower (detergent rich) phases were subjected to dot blotting. A non-reconstituted protein (control sample) was subjected to the same procedure.

3. Results

3.1. Langmuir balance studies: protein adsorption at the air–water interface

SCRΔ tendency to self-aggregate strongly suggests that the protein is a surface-active molecule. This is confirmed by its behavior in the Langmuir balance. Langmuir balance approaches are based on the measurement of the water surface tension by means of a suspended solid probe (the Wilhelmy plate) that is slightly introduced into the air–

liquid interface. Injection of the mutant in the aqueous phase led to a rapid, dose dependent increase of lateral pressure π at the air–water interface. π is usually measured in nN/m. An increase in π is an indication that the protein is adsorbing onto the air–water interface. Time courses of interface adsorption of SCRΔ at different concentrations can be seen in Fig. 1A. A discontinuity in the π vs. time plots was observed for all SCRΔ concentrations, that could be attributed to a small change in protein conformation, but the phenomenon was not further explored.

Fig. 1C–D summarizes the change in Δπ as a function of SCRΔ and MBP–SCR protein concentrations, respectively. For the mutant protein, the increase in surface pressure appears to reach a plateau value of ~24 mN/m at 100 nM concentration. For the MBP–SCR fusion protein the increase in surface pressure is lower than in the case of SCRΔ, with a plateau value of ~15 mN/m (at 25–50 nM), presumably due to the stabilizing effect of the bound MBP.

SCR cannot be separated from MBP as a result of its high hydrophobicity and instability in the absence of detergents. Pure MBP gave a signal of incorporation to the air–water interface (Fig. 1B), but it was found later that it did not interact with lipid monolayers (see below). Hence, surface pressure changes when working with lipid monolayers are due to the SCR moiety, without any signal component arising from MBP.

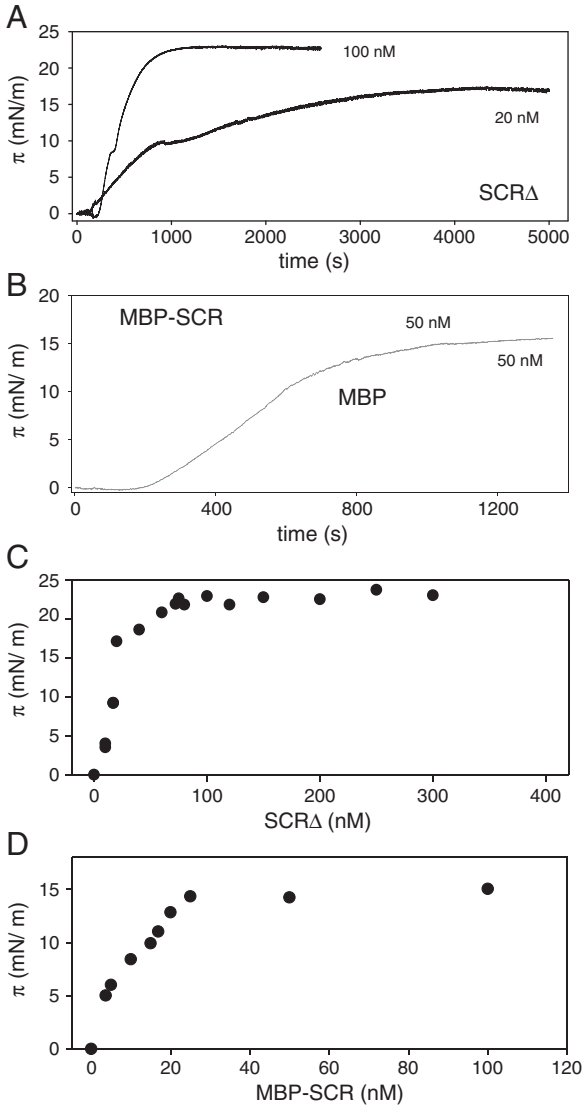

Fig. 1. Langmuir balance measurements at the air–water interface. (A, B) Time course of adsorption of SCRΔ (A) and MBP–SCR (black) and MBP (grey) (B) at the air–water interface. On (C, D) the maximum increase in lateral pressure caused by SCRΔ (C) and MBP–SCR (D) adsorption at the air–water interface is shown. The change in lateral pressure is plotted as a function of initial protein concentration injected to the subphase.

3.2. Langmuir balance studies: protein insertion into lipid monolayers

When a monomolecular film of phospholipid is spread at the lipid–water interface, once equilibrium is reached an initial lateral pressure π_i is measured for the lipid monolayer. Protein insertion into the monolayer will increase the lateral pressure by Δπ. SCRΔ (400 nM injected to the subphase) was able to insert into lipid monolayers of PC extended at the air–water interface (Fig. 2A). The same trend was observed for MBP–SCR (100 nM injected to the subphase) (Fig. 2B). However MBP alone did not become inserted into the lipid monolayer (Fig. 2B).

Fig. 3 shows the change in lateral pressure as a function of monolayer initial pressure, for SCRΔ inserted in PC and PC:PS monolayers (Fig. 3A) and for MBP–SCR inserted in PC and PC:PS monolayers (Fig. 3B). The protein insertion-dependent change in lateral pressure (Δπ) decreases linearly for both proteins with the increase in initial pressure (π_i). In the case of MBP–SCR (Fig. 3B) the presence of PS does not clearly modify the protein interaction with the lipid monolayer. On the contrary for SCRΔ (Fig. 3A) PS affects protein insertion, decreasing somewhat the protein capacity to interact with the monolayer.

Above a certain π_i value (termed critical pressure or π_c) no more protein insertion is observed. π_c can be calculated by extrapolating the Δπ vs. π_i line to $\Delta\pi = 0$. In the case of SCRΔ (Fig. 3A), the obtained π_c values are within the accepted average value of 30 mN/m ± 5 mN/m for the lateral pressure of cell membranes [16–18]. The π_c calculated values for MBP–SCR (Fig. 3B) are at the lower limit of membrane insertion, probably due to the stabilizing effect of MBP on protein structure, which could decrease its affinity for lipids (see Table 1). In general the data suggest that SCRΔ could insert into model or cell membranes.

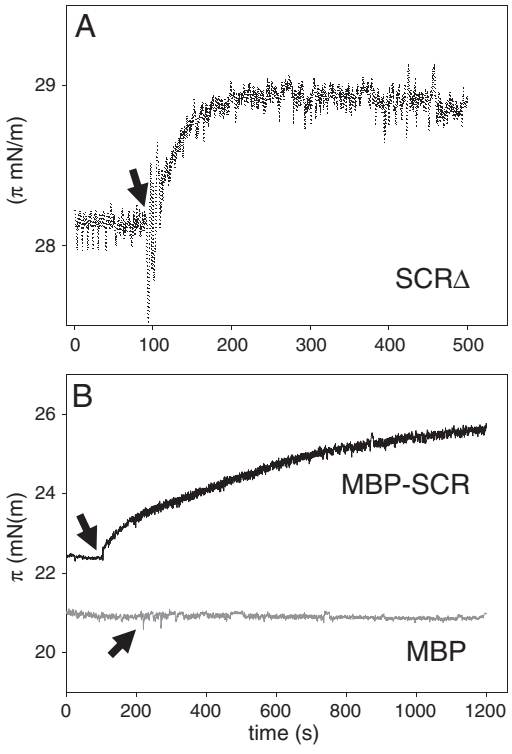

Fig. 2. Time course of adsorption of SCRΔ (A), and MBP–SCR (black) and MBP (grey) (B) at the PC–water interface. The arrow indicates the time of protein addition to the subphase.

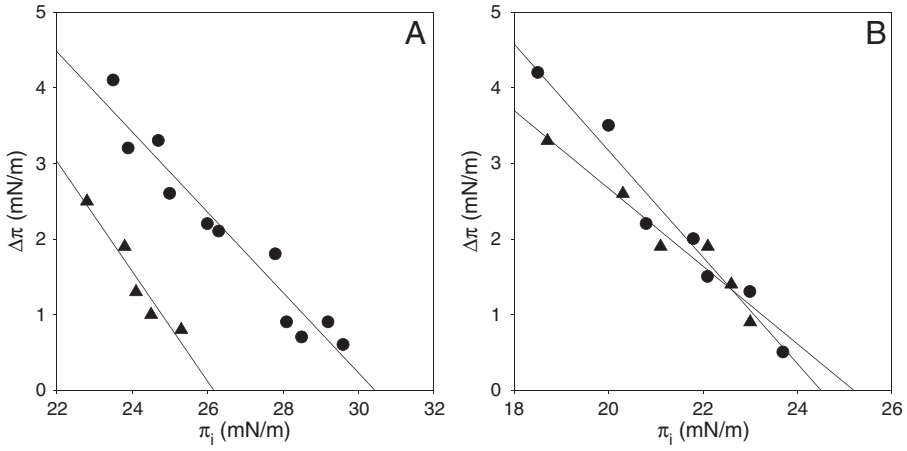

Fig. 3. Maximum increase in lateral pressure after SCRΔ (A) and MBP–SCR (B) insertion in lipid monolayers. The appropriate lipids were spread as a monomolecular film at the air–water interface, at a lateral pressure π_i. Then proteins were injected into the subphase and the increase in lateral pressure $\Delta\pi$ was recorded. Lipids were: [●] PC, [▲] PC:PS (9:1 mol ratio). Data reported as a function of initial lateral pressure.

3.3. The SCR cytoplasmic domain binds model membranes

The basis of the centrifugation method for measuring SCRΔ and MBP–SCR proteins binding to LUV is shown in Figs. 4 and 5, respectively. When either vesicles or protein are independently centrifuged in D_2O buffer, the lipid remains on top of the tube while all of the protein sediments at the tube bottom. But when LUVs and protein are incubated together and the mixture centrifuged, a substantial part of the protein remains in the top part of the tube, i.e. vesicle-bound [12].

Figs. 4A and 5A display the dot blots developed for this test for each protein (SCRΔ and MBP–SCR, respectively) and show that a protein fraction could always be extracted from the tube wall irrespective of the presence of vesicles. However, in all cases the proteins incubated with LUVs remained partly in solution, presumably associated to the vesicle membrane.

In the case of SCRΔ (Fig. 4) the protein is seen to bind LUVs, as compared to the pure protein which, in the absence of vesicles, sediments at the tube bottom. In the case of MBP–SCR (Fig. 5) protein binding to LUVs is similar to that observed for SCRΔ (protein:lipid ratios indicated in the corresponding figure legends). In none of these cases protein binding led to vesicle aggregation or vesicle content efflux (data not shown).

3.4. Analysis of the reconstitution profile

In a further effort to characterize the nature of the interaction of SCR cytoplasmic domain (SCRΔ) with lipid bilayers, we attempted the solubilization and phase separation in Triton X-114 of free and membrane-bound proteins, the latter having been previously reconstituted in LUVs. Proteoliposomes were isolated from the non-bound protein by D_2O ultracentrifugation (Fig. 6) and the 2 upper aliquots (numbered 1 and 2) were combined for the solubilization assay. Note that the lipid-protein ratios of the reconstituted proteoliposomes were lower than those measured directly on the lipid-protein mixtures (Figs. 4 and 5).

The detergent Triton X-114 forms a homogeneous phase at 4 °C, but separates into detergent-poor and detergent-rich phases above 20 °C [13]. Proteins solubilized at 4 °C can therefore be separated by rising the temperature: the hydrophilic proteins or proteins which do not strongly interact with the vesicles are found exclusively in the detergent-poor phase, whereas integral membrane proteins with a strong amphipathic nature are recovered in the detergent-rich phase. SCRΔ and MBP–SCR, either free or reconstituted, were solubilized in 1% TX-114 (4 °C) and then incubated at 30 °C, subsequent detergent phase separation being helped by low-speed centrifugation. Aliquots of detergent-rich and detergent-poor fractions were analyzed by dot blot (Fig. 7), and the corresponding controls (pure proteins, no LUVs) were included for a direct comparison.

In the absence of lipids, the proteins appeared both in the detergent-poor (upper) and detergent-rich (lower) phases (Fig. 7A–C) probably due to their amphipathic nature. However, when proteins were previously reconstituted in PC:PS (9:1) LUVs, in both cases the detergent-poor phase was virtually devoid of protein, which was only found in the detergent-rich phase (Fig. 7B for SCRΔ and 7D for MBP–SCR), thus confirming that the protein cytoplasmic portion, even in the absence of the protein TMD, interacts strongly with lipids, a behavior that reminds that of intrinsic proteins.

Table 1
Critical pressures (π_c) for SCRΔ and MBP–SCR insertion in monolayers. Data calculated from the graphics in Fig. 3. The tendency line associated standard error is given for each π_c.

Protein	PC	PC:PS 9:1 (mol:mol)
SCRΔ	30.4 mN/m (±0.31)	26.2 mN/m(±0.22)
MBP–SCR	24.4 mN/m (±0.28)	25.1 mN/m(±0.22)

4. Discussion

The present study focuses on a truncated form of SCR, consisting of the protein cytoplasmic portion. It was previously seen [3,4] that removal of the TMD domain totally inhibited its activity as a lipid flip-flop catalyst, perhaps by virtue of the decreased calcium-affinity constant (5-fold). Here we intended to analyze the TMD suppression impact on the protein lipid affinity (mono- and bilayers).

It should be noted that the proteins used in this study have been expressed in *E. coli*, thus they do not contain the post-translational modifications, mainly palmitoylation, that are known to occur in mammalian cells. However the absence of acylation does not abolish the scramblase activity [3,4,21], suggesting that the lack of acyl residues does not lead to major changes in the protein structure/function. Moreover Merregaert et al. [11] observed that native SCR expressed in HaCaT cells in the presence of 2-bromopalmitate, thus in the absence of acylation, is localized to the plasma membrane and secreted to the extracellular medium. These authors conclude that acylation is not involved in intracellular membrane traffic. An additional factor to be considered when interpreting the above results is that the C-terminal domain of SCRΔ may be incorrectly folded, allowing the exposure of hydrophobic patches to the aqueous medium. However, according to ANS binding experiments [3] this appears to occur to a moderate extent. In general the available data support the validity of our observations with *E. coli*-expressed SCRΔ.

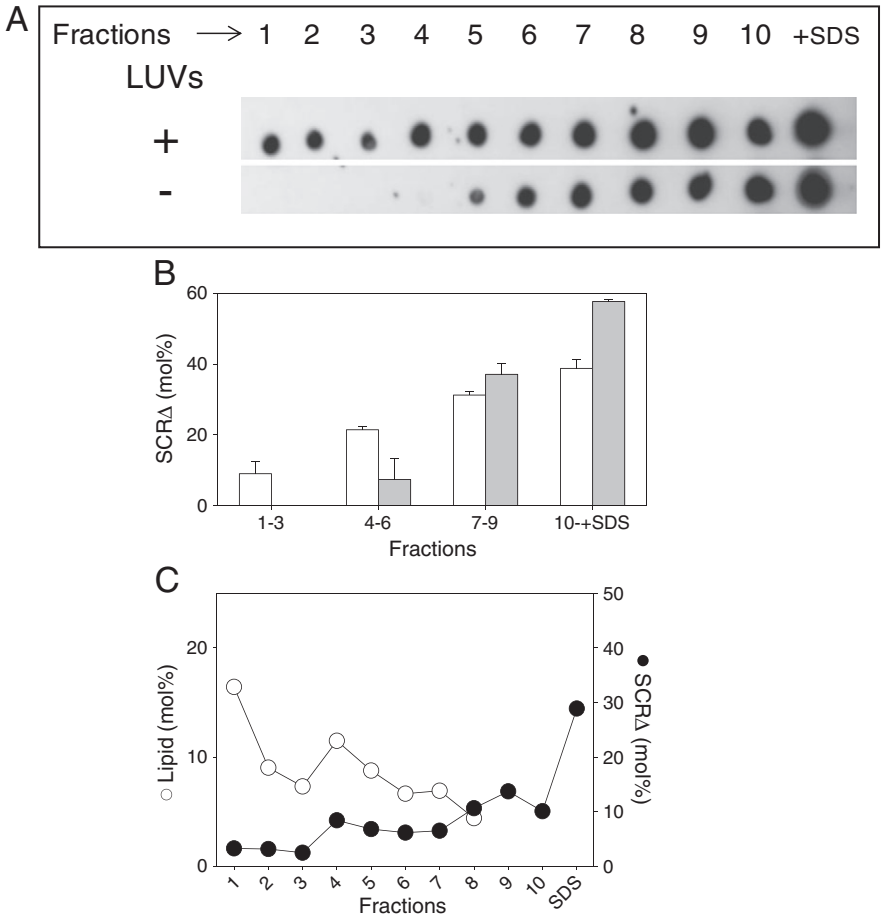

Fig. 4. Direct measurements of SCRΔ binding to PC:PS (9:1) phospholipid vesicles. In (A) dot blot of the recovered samples is shown (representative dot blot of two independent experiments), when protein was incubated in the absence/presence of LUVs. (B) recovered pure protein (grey) and protein incubated with LUVs (white) are shown. In (C) protein (●) incubated with lipid in the form of LUVs (○) is represented. All data were normalized to the total protein and lipid recovered (mol%) (average of n = 2 independent experiments). The initial protein: lipid ratio was 1:1000 (mol:mol). The protein:lipid ratio of the SCRΔ bound fractions (average of the top 4 layers) is: [1,2] 1:3900; [3,4] 1:2100.

4.1. SCRΔ interacts with lipid monolayers

Fig. 1A–C shows the adsorption of SCRΔ at the air–water interface, demonstrating the surface-active nature of this protein fragment. Monolayer studies have been extensively used in the study of purified membrane proteins interacting with lipid layers [16–18], and this was also performed with the truncated SCR. By spreading pure PC or PC mixtures with PS (9:1 mol:mol) the affinity for lipids was analyzed. SCRΔ may become inserted into monolayers at an initial π_0 in the range 26–30 mN/m (Fig. 3A). This is important because cell membranes are considered to support a lateral pressure $\pi \approx 30$ mN/m [16], albeit with large fluctuations around this average value (± 5 mN/m). Although the lateral pressures obtained for the mutant membrane protein are below those found for other proteins in the Langmuir balance, e.g. the toxins equinatoxin II ($\pi_c \sim 50$ mN/m) [19] or *E. coli* α-hemolysin ($\pi_c \sim 35$ mN/m) [20], the data in Fig. 3 indicate that SCRΔ can insert into model or cell membranes, demonstrating the surface-active nature of the scramblase cytoplasmic domain.

4.2. SCRΔ binds vesicles

The data on protein interaction with LUV (Figs. 4 and 5) demonstrate that both proteins MBP–SCR and SCRΔ bind lipid bilayers. Note however that both proteins are differently purified: SCRΔ is more stable in buffer in the absence of the TMD as compared to native SCR, and can be obtained at low concentrations in the absence of aggregation. For the wild type it is not possible to separate the SCR portion from its partner (MBP) unless when reconstituted or in the presence of detergent. The MBP stabilizing effect is clear (Fig. 5): the wild type protein binding to LUVs might be weaker than SCRΔ binding to LUVs, since MBP allows SCR stability in solution thus SCR does not require LUVs binding for stability. On the contrary, the SCRΔ amphipathic nature is remarkable, and the absence of TMD does not hinder the protein strong binding to LUVs (Fig. 4).

4.3. SCRΔ behaves as an intrinsic protein

MBP–SCR and SCRΔ were subjected to the same reconstitution process. The fraction patterns isolated from the D_2O ultracentrifugation were very similar (Fig. 6), as compared with the pure non-reconstituted proteins. Nevertheless, SCRΔ could be either just adsorbed or strongly bound to the vesicle surface (Fig. 4), so an additional test was performed, to clarify the mutant precise interaction with LUVs.

TX-114 has been shown to solubilize membranes, allowing a clear phase separation, leaving the soluble and the extrinsic membrane proteins in the detergent-poor phase, and with the detergent-rich phase containing the integral membrane proteins [13–15]. The latter is the way in which both proteins, MBP–SCR and the TMD-lacking mutant, behave (Fig. 7).

4.4. The properties of MBP–SCR

The instability of intrinsic and other strongly membrane-bound proteins in aqueous media makes essential the use of stabilizing techniques,

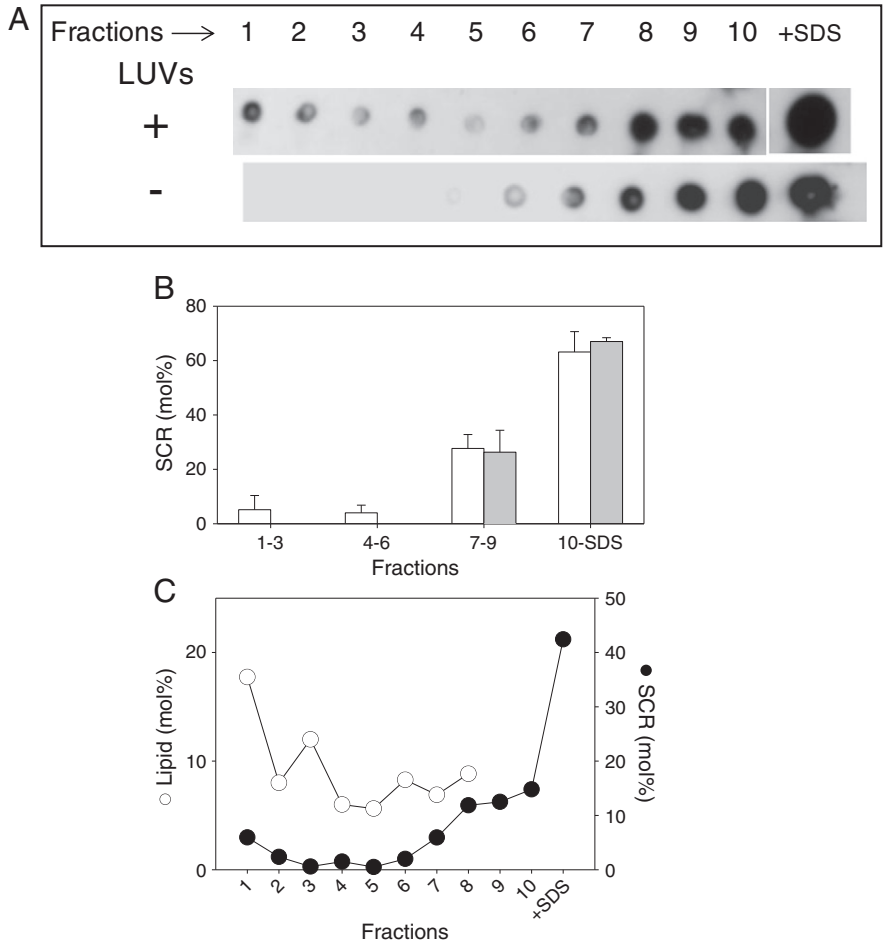

Fig. 5. Direct measurements of MBP–SCR binding to PC:PS (9:1) phospholipid vesicles. In (A) dot blot of the recovered samples is shown (representative dot blot of two independent experiments), when protein was incubated in the absence/presence of LUVs. (B) recovered pure protein (grey) and protein incubated with LUVs (white) are shown. In (C) protein (●) incubated with lipid in the form of LUVs (○) is represented. All data were normalized to the total protein and lipid recovered (mol%) (average of n = 2 independent experiments). The initial protein:lipid ratio was 1:1000 (mol:mol). The protein:lipid ratio of the MBP–SCR bound fractions (average of the top 4 layers) is: [1,2] 1:3100; [3,4] 1:12,000.

of which protein isolation in detergent environments is probably the most widely used strategy. However the use of detergents, even in minute proportions, can perturb the results of techniques used in the study of lipid–protein interactions. As an alternative, a MBP–SCR fusion protein has been used in this study, with the MBP moiety bound to the amino end of SCR, thus away from the putative TMD located at the carboxyl end. The results are interesting because the protein is stable in detergent-free buffers (data not shown) and yet it binds lipid monolayers (Fig. 3B) and bilayers (Fig. 5), something that free MBP cannot do (Fig. 2B). MBP–SCR can be reconstituted in bilayers (Fig. 6) and behaves thereafter as an intrinsic protein (Fig. 7). Additionally, MBP does not hamper scramblase activity when anchored to the SCR N-ter (data not shown). Thus the data appear to reinforce that the C-terminal TMD is essential for the scramblase activity.

4.5. SCR TMD and cytoplasmic domain interaction with membranes

Even when SCRΔ shows such a high affinity for lipids (Figs. 3, 4 and 6), it cannot promote lipid randomization at the assayed calcium concentrations [3,4], confirming that the SCR cytoplasmic domain is not enough for the promotion of lipid translocation. Thus the TMD appears to be essential for the scramblase activity. Our preliminary data (not shown) suggest that reconstituted MBP–SCR fusion protein is able to promote similar pyrene-SM flip-flop as SCR, in agreement with Zhou et al. [21], therefore the MBP anchor at the N-terminus of SCR does not hamper SCR translocation mechanism, as long as there is a TMD.

TMD, apart from its role as SCR anchor to the cell membrane, might be the putative segment F of the EF-hand motif [22,23], although several researchers disagree on this matter [10,24]. Calcium binding could change the folding or the tilting of the protein TMD (apart from changing the tertiary structure of the whole protein cytoplasmic domain), this new conformation allows the membrane lipid scrambling activity. In fact, the presence of transmembrane stretches of proteins in the bilayer is known to be sufficient to allow some degree of phospholipid flip-flop in membranes [25–27]. For instance, Langer et al. [28] confirmed that peptides mimicking the α-helices of SNARE protein TMD could stimulate lipid flipping in model membranes, although they could not promote vesicle fusion as wild type SNARE protein complexes do.

In view of the uncertainties surrounding the role of TMD in the SCR activity, studies have been undertaken in our laboratory in which synthetic TMD-mimetic peptides have been examined in the presence of lipid bilayers [5], showing that the TMD can be inserted in lipid bilayers of various compositions. Moreover, experiments reported here support that the N-terminal cytoplasmic moiety of SCR is also an important determinant for protein binding to model membranes. Thus in the cell environment, both the SCR cytoplasmic and transmembrane domains may feature flexible and adaptive interactions with the membrane. It should also be remembered that native SCR, but not the protein overexpressed in bacteria used in this kind of studies, is multipalmitoylated

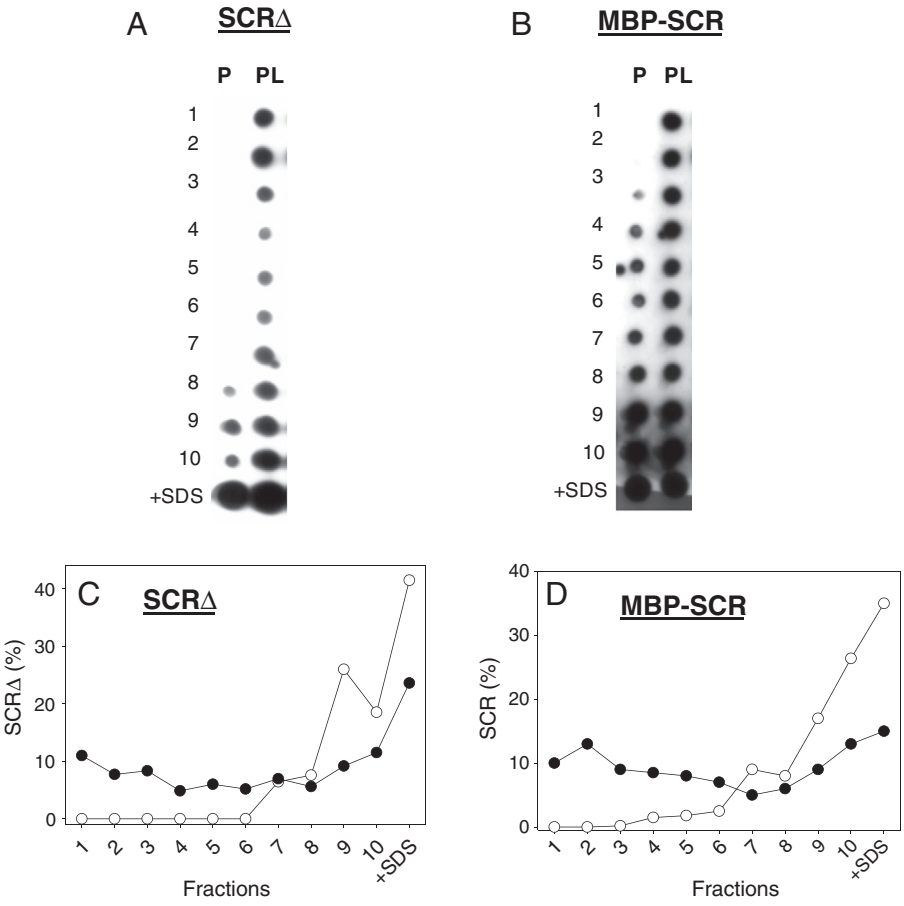

Fig. 6. Reconstitution of SCRΔ (A–C) and MBP–SCR (B–D) into PC:PS vesicles and analytical isolation in D_2O ultracentrifugation. (A, B) Dot blots of the recovered samples, "P" for pure protein and "PL" for proteoliposome sample. (C, D) protein quantitation (mol%), (○) for pure protein and (●) for protein subjected to the reconstitution procedure (average of n = 2 independent experiments). The protein:lipid ratio of the protein bound fractions (top 2 layers) is: SCRΔ 1st – (1:2,800); 2nd – (1:2500). MBP–SCR 1st – (1:2100); 2nd – (1:900).

[25], so that the lipid anchors will further contribute to docking the protein to the cell membrane.

We conclude that the available evidence for the interaction of SCR with membranes points to a model in which the putative TMD would get inserted into the bilayer as in a classical monotopic protein, while the major cytoplasmic domain would interact directly and perhaps also through acyl chains with the membrane lipid matrix.

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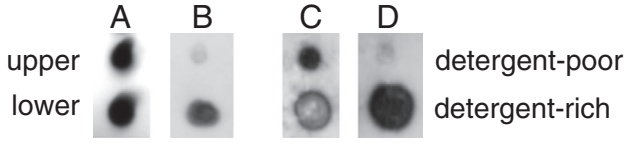

Fig. 7. Solubilization with Triton X-114. Pure SCRΔ (A), pure MBP–SCR (C), reconstituted SCRΔ (B) and reconstituted MBP–SCR (D) solubilization by Triton X-114. The lower/upper ratios of values in densitometric units are: A = 1.3, B = 10.4; C = 0.8, D = 15.4.

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