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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–