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Membrane binding and insertion of the predicted transmembrane domain of human scramblase 1

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ABSTRACT

Human phospholipid scramblase 1 (SCR) was originally described as an intrinsic membrane protein catalyzing transbilayer phospholipid transfer in the absence of ATP. More recently, a role as a nuclear transcription factor has been proposed for SCR, either in addition or alternatively to its capacity to facilitate phospholipid flip-flop. Uncertainties exist as well from the structural point of view. A predicted α-helix (aa residues 288–306) located near the C-terminus has been alternatively proposed as a transmembrane domain, or as a protein core structural element. This paper explores the possibilities of the above helical segment as a transmembrane domain. To this aim two peptides were synthesized, one corresponding to the 19 α-helical residues, and one containing both the helix and the subsequent 12-residues constituting the C-end of the protein. The interaction of these peptides with lipid monolayers and bilayers was tested with Langmuir balance surface pressure measurements, proteoliposome reconstitution and analysis, differential scanning calorimetry, tests of bilayer permeability, and fluorescence confocal microscopy. Bilayers of 28 different lipid compositions were examined in which lipid electric charge, bilayer fluidity and lateral heterogeneity (domain formation) were varied. All the results concur in supporting the idea that the 288–306 peptide of SCR becomes membrane inserted in the presence of lipid bilayers. Thus, the data are in agreement with the possibility of SCR as an integral membrane protein, without rejecting alternative cell locations.

1. Introduction

Cell membranes are composed of an asymmetric lipid bilayer in which proteins are embedded. Some of these proteins, named flippases and floppases, are in charge of maintaining the transbilayer phospholipid asymmetry [1,2], with PS predominantly in the inner leaflet. When cells are challenged, in processes such as blood coagulation or apoptosis, intracellular calcium increases accompanied by PS exposure to the outer leaflet via an ATP-independent pathway [3]. The first described member of the phospholipid scramblase family, human phospholipid scramblase 1 (SCR) seems to be the main protein responsible for this event [4,5]. This multifunctional protein is a type-2 membrane protein of 318 aa. It has been described as a lipid raft-associated protein when multipalmitoylated [6,7]. It has been predicted that SCR contains an N-terminus (1–287 aa) cytoplasmic main portion, a putative transmembrane α-helix (288–306 aa), and a small C-terminus extracellular coil (307–318 aa) [8]. Protein-, lipid- and DNA-binding networks connected to SCR are abundant, since posttranslational acylation acts as a switch controlling the scramblase localization. In the absence of acylation, scramblase 1 is imported into the nucleus where it binds DNA and acts as a transcription factor [9,10].

The nature of the physiological activity of SCR in the cell remains controversial. Its role as a scramblase has been challenged due to its involvement in seemingly unrelated events in cell signaling [9,10]. Also relevant in this discussion is the recent identification of TMEM16F as the responsible protein for the defective phospholipid scrambling in Scott syndrome when truncated [11]. The situation is not better understood from the structural point of view. Sahu et al. [12] found that the EF hand-like calcium-binding domains of the scramblase family showed a marked deviation from the classical sequence, and suggested a novel class of low affinity calcium-binding domains. The scramblase anchoring to the membrane has also been challenged [13] due to high similarities in the sequence with the crystallized homologous At5g01750 from *Arabidopsis thaliana* and also with Tubby-like proteins, presuming that the highly hydrophobic α-helical domain, sometimes considered as a transmembrane domain, might remain buried in the protein core with the palmitoyl residues as the only tether to the bilayer. However, in a recent paper, Francis et al. [14] have provided evidence, based on fluorescence quenching studies, that the C-terminal α-helix inserts into membranes.

Abbreviations: ANTS, 8-aminonaphtalene-1,3,6-trisulfonic acid sodium salt; DID, 1,1′-dioctadecyl-3,3,3′,3′-tetramethyl-indodicarbocyanine; DPX, p-xylene-bis(pyridinium) bromide; GUVs, giant unilamellar vesicles; HFIP, 1,1,1,3,3,3-hexafluoro-2-propanol; LUVs, large unilamellar vesicles; MLVs, multilamellar vesicles; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; pSM, palmitoyl sphingomyelin; SCR, human phospholipid scramblase 1, or hPLSCR1; TM, transmembrane; TM19, SCR 288–306 peptide; TM31C, SCR 288–318 peptide

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In order to shed some light on the SCR interaction with membranes two peptides were synthesized, one consisting of the putative transmembrane domain, TM19 [^{288}KMKAVMIGACFLIDFMFFE^{306}] and a second one containing the TM domain plus the exoplasmic coil, TM31C [^{288}KMKAVMIGACFLIDFMFFESTGSQEQKSGVW^{318}] (Fig. 1). No palmitoylation occurs in this part of the protein under physiological conditions. Using a set of well-established biophysical approaches, we studied the interactions of each peptide with model membranes of differing charges and/or phase structures. The results in this paper, together with published topological predictions, give strong support to the notion that the 288–306 peptide of SCR constitutes a transmembrane domain rather than existing inside the protein core.

2. Materials and methods

2.1. Chemicals

TM31C and TM19 were synthesized and purchased from PolyPeptide Group Laboratories (Strasbourg, France), and stored at −20 °C in powder form. When required, they were dissolved in DMSO (Sigma) or HFIP (Fluka). Egg phosphatidylcholine (PC), spinal cord phosphatidylserine (PS), egg phosphatidylethanolamine (PE), egg phosphatidylglycerol (PG) and liver phosphatidylinositol (PI) were purchased from Lipid Products (Redhill, England). The remaining lipids were all from Avanti Polar Lipids (Birmingham, AL, USA). 8-Aminonaphtalene-1,3,6-trisulfonic acid sodium salt (ANTS), p-xylene-bis(pyridinium) bromide (DPX) and 1,1′-dioctadecyl-3,3,3′,3′-tetramethyl-indodicarbocyanine (DID) were obtained from Invitrogen (Life Technologies, Carlsbad, CA, USA). The polyclonal anti-scramblase antibody was from Oncogene (Cambridge, UK). FITC-linked anti-rabbit antibody was from Abcam (Cambridge, UK) and HRP-linked anti-rabbit antibody was from New England Biolabs (Ipswich, MA, USA). All other reagents were of analytical grade. Assay buffer was 10 mM Hepes, 150 mM NaCl, pH 7.4.

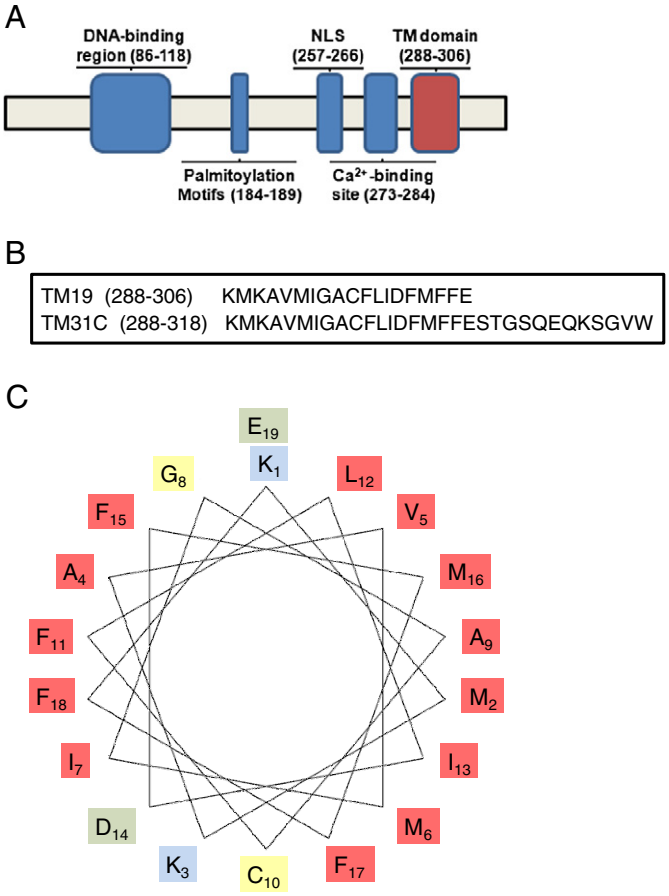

Fig. 1. The structure of human SCR and of the putative transmembrane domain. (A) Human SCR main domains, the predicted transmembrane helix at the C-end is marked in red. (B) The two peptides used in this work, TM19, comprising the transmembrane domain, and TM31C, which includes the TM domain plus the extracellular coil. (C) Wheel diagram of TM19, hydrophobic residues are marked in red.

2.2. Langmuir balance measurements

Peptide-induced changes in surface pressure at the air–water interface and peptide–lipid monolayer interactions were studied at 25 °C using a 1.25 ml multi-well Delta Pi-4 Langmuir balance (Kibron Inc., Helsinki, Finland). 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 attained. The peptides dissolved in DMSO (less than 0.5% of total volume) were injected with a micropipette through a hole connected to the subphase, and their surface activity followed by means of surface pressure changes with constant stirring.

2.3. Peptide binding quantification

The appropriate amounts of peptide and LUVs were co-incubated at a lipid-to-peptide ratio 75:1 for 2 h at 25 °C in a ThermoMixer (Eppendorf, Hamburg, Germany). Sucrose gradient formation and ultracentrifugation were carried out as in [15]. A fraction of this sample was adjusted to a 1.4 M final sucrose concentration (final volume 300 μl), overlaid with 400 μl 0.8 M sucrose in buffer, and 300 μl 0.5 M sucrose. The gradient was centrifuged at 400,000 ×*g* for 3 h, and then four 250 μl fractions were collected from the bottom of the tube with a Hamilton syringe. The polycarbonate centrifuge tubes were then washed with 250 μl hot 1% (w/v) SDS to recover the peptide that had aggregated or adhered to the tube walls.

Dot blots were performed using a Hybond-C Extra (Amersham Biosciences) membrane. The sucrose gradient-derived samples were spotted onto the membrane and blocked with 5% skim milk for 1 h, followed by 1 h incubation with anti-scramblase antibody (1:400). The blot was washed several times with PBS, pH 7.4, and incubated for 1 h with an HRP-linked anti-rabbit 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).

2.4. Differential scanning calorimetry

All measurements were performed using a VP-DSC high-sensitivity scanning microcalorimeter (Microcal, Northampton, MA, USA). For peptide–MLV preparation, the proper amounts of lipid in chloroform:methanol (2:1, v/v) and peptide in HFIP were mixed and the solvents evaporated exhaustively. The MLVs were then prepared by slowly hydrating the peptide-containing lipid film with assay buffer at a temperature above the lipid phase transition temperature, continuously stirring with a glass rod and with vigorous vortexing. The samples were then carefully degassed prior to the measurements. Assay buffer was scanned as a background. The scan rate was 45 °C/h. Samples were scanned several times to ensure the reproducibility of the endotherms. Data were analyzed using ORIGIN software provided by MicroCal. Final volume and lipid concentration in the cell were 0.5 ml and 0.5 mM respectively. Lipid concentration was measured as lipid phosphorous using a molybdate reagent.