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The C-terminal transmembrane domain of human phospholipid scramblase 1 is essential for the protein flip-flop activity and Ca^{2+}-binding

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Abstract Human phospholipid scramblase 1 (SCR) is a 318 amino acid protein that was originally described as catalyzing phospholipid transbilayer (flip-flop) motion in plasma membranes in a Ca^{2+}-dependent, ATP-independent way. Further studies have suggested an intranuclear role for this protein in addition. A putative transmembrane domain located at the C terminus (aa 291–309) has been related to the flip-flop catalysis. In order to clarify the role of the C-terminal region of SCR, a mutant was produced (SCRΔ) in which the last 28 amino acid residues were lacking, including the α-helix. SCRΔ had lost the scramblase activity and its affinity for Ca^{2+} was decreased by one order of magnitude. Fluorescence and IR spectroscopic studies revealed that the C-terminal region of SCR was essential for the proper folding of the protein. Moreover, it was found that Ca^{2+} exerted an overall destabilizing effect on SCR, which might facilitate its binding to membranes.

Keywords Transmembrane lipid motion · Phospholipid scramblase 1 · Calcium binding protein

Introduction

Phospholipid scramblases (Sahu et al. 2007) constitute a group of homologous bidirectional lipid translocators that are conserved in all eukaryotic organisms (Contreras et al. 2010). In humans, four related scramblase proteins have been identified (Wiedmer et al. 2000) and more recently a fifth one has been identified at the transcript level. Furthermore Suzuki et al. (2010) described calcium-dependent phospholipid scrambling by a transmembrane protein (TMEM16F) in animal cells, the protein being putatively related to the Scott syndrome.

The first described member and prototype of the family is the human phospholipid scramblase 1 (SCR), a 318 aa, type II endofacial membrane protein, expressed in a variety of hematologic and non hematologic cells. SCR remains inactive under normal conditions, but increased cytosolic calcium concentrations (≈1,000-fold the basal level in response to cell injury, coagulation, cell activation, or apoptosis) activate the protein and induce transbilayer movement of phospholipids across the membrane, destroying the bilayer asymmetry (Bassé et al. 1996; Zhou et al. 1997; Zhao et al. 1998a). The actual cellular functions of SCR remain somewhat unclear, because in addition to its putative role in mediating transbilayer movement of phospholipids, SCR may also regulate at the nuclear level processes including signaling, cell proliferation and differentiation, apoptosis, injury, transcription, antibacterial, and antiviral activities (Sun et al. 2002; Ben-Efraim et al. 2004; Zhou et al. 2005; Wyles et al. 2007; Amir-Moazami et al. 2008; Smrz et al. 2008; Kuo et al. 2011; Lizak and

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Yarovinsky 2012; Yang et al. 2012; Kusano and Eizuro 2013).

SCR is a palmitoylated protein that was found to partition with the EGF receptor in lipid rafts. EGF stimulates tyrosine phosphorylation of SCR and promotes protein internalization (Sun et al. 2002). In the absence of palmitoylation, virtually all of the expressed SCR localizes to the nucleus (Wiedmer et al. 2003). 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 plasma membrane (Zhao et al. 1998a; Wiedmer et al. 2003). A structural model computed by homology modeling suggests that palmitoylation may represent the principal membrane anchorage for the SCR (Bateman et al. 2009). Hydrolysis of the thioester-bound palmitoyl groups in SCR purified from RBC markedly reduced the scrambling activity (Zhao et al. 1998b; Wiedmer et al. 2003), and the unmodified SCR expressed and purified from *Escherichia coli* had approximately 50 % of the activity observed for the endogenous protein purified from RBC (Zhou et al. 1997).

An important region in the protein is the calcium binding motif (aa 273–284) with a putative EF-hand-like structure essential for the scrambling activity of SCR. This motif is known to bind calcium and other ions. In this motif, residues in positions 1, 3, 5, 7, 9, and 12 contribute to the octahedral coordination of the calcium ion and substitution at any of these positions reduces phospholipid scramblase function. Calcium binding promotes conformational changes in the protein structure that include protein self-aggregation (Stout et al. 1998; Zhou et al. 1998). Similar conformational changes were observed by Francis and Gummadi (2012) for Scramblase 4 in the presence of calcium. A structural model of SCR suggests that the calcium binding motif is not an EF-hand like structure (Bateman et al. 2009). Studies of Sahu et al. (2009) indicate that the calcium-binding motif present in scramblase represents a new class of low-affinity calcium binding motifs, "the scramblase-like calcium binding motif". This motif would also be present in unrelated proteins that are known to exhibit changes in biochemical parameters upon binding of calcium and other divalent ions.

SCR has also a predicted inside-to-outside oriented transmembrane helix (aa 291–309), and on this basis SCR is classified as a type II membrane protein. Finally, the remaining chain portion (aa 310–318) is considered as a short exoplasmic tail (Zhou et al. 1997; Sims and Wiedmer 2001). In contrast to this model, Bateman and coworkers provide an alternative SCR model in which the α-helix instead of being inserted into the membrane is buried within the protein core (Bateman et al. 2009). In a recent paper Francis et al. (2013) suggest that the C-terminal helix is required for membrane insertion and scrambling activity.

In order to clarify the role of the C-terminal α-helix in the structure–function relationship of SCR we constructed a truncated mutant (SCRΔ), that lacks the C-terminal 28 aa (α-helix + C-terminal peptide) (Fig. 1). A combination of structural and functional studies (fluorescence and infrared spectroscopies, partial trypsin digestion, and functional characterization using liposomes) reveal that the α-helix is crucial for the scramblase activity and for a correct protein folding and stability. Furthermore, in the presence of calcium the truncated form SCRΔ binds the ion and undergoes conformational changes, but needs higher Ca^{2+} concentrations than the wild type. Finally calcium binding decreases the stability of SCR, perhaps in this way increasing its capacity to bind membrane lipid bilayers.

Methods

Materials

Egg phosphatidylcholine (PC) and spinal cord phosphatidylserine (PS) were purchased from Avanti Polar Lipids (Alabaster, AL) and Lipid Products (South Nutfield, UK), respectively. 1-Lauroyl-2-(1-pyrenebutyroyl)-sn-glycero-3-sphingomyelin (py-SM) was synthesized as described in Müller et al. (2000), 1-hexadecanoyl-2-(1-pyrenedecanoyl)-*sn*-glycero-3-phosphocholine (py-PC) and ANS were supplied by Invitrogen and all restriction enzymes, Factor Xa and Amylose resin were from New England BioLabs (Ipswich, MA, USA). Trypsin (swine, expressed in *Pichia pastoris*) was of proteomics grade from Roche (Mannheim, Germany). The monoclonal anti-scramblase antibody was from Abcam (Cambridge, UK) and HRP-linked anti-mouse antibody was from New England Biolabs. All other reagents were of analytical grade.

Construction of SCRΔ

The mutant SCRΔ was obtained by PCR amplification of the SCR fragment including the DNA sequence corresponding to residues M1-K290, using as primers two oligonucleotides, that contain in their sequences *Nde*I (A) and *Bam*HI (B) restriction sites, respectively:

A. 5′-ATTTCA**CATATG**GACAAACAAAACTCACAG ATG-3′
B. 5′-GCTTGC**GGATCC**CTATTTCATTTTAACATCA AGGTCTAA-3′

The fragment was then inserted in the expression vector pET-15b, so that the mutant was expressed with a N-terminal His tag sequence.

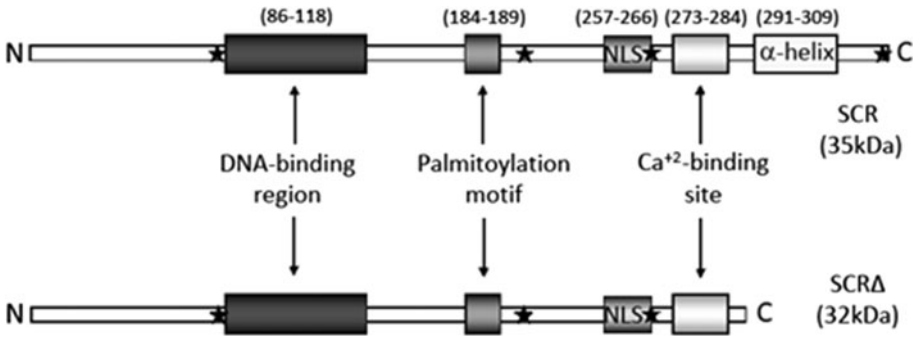

Fig. 1 Schematic representation of the sequences of native SCR and the truncated mutant SCRΔ. (*filled star*) Trp residues (85, 210, 263, 318)

Cloning of SCR

The SCR fragment was amplified by PCR using the primers A and C that contain in their sequences *Nde*I (A) and *Bam*HI (C) restriction sites, respectively:

C. 5′-GCTTGC**GGATCC**CTACCACACTCCTGATTTTT GTTCC-3′

SCR was then inserted in the pMAL-c5X vector modified with a His tag at the N-terminal MBP sequence. The vector was kindly donated by J.C. Karam and S. Mañes (CNB, Madrid). All the cloned inserts were further confirmed by nucleotide sequencing.

Purification of wild-type and mutant SCR proteins

The SCRΔ purification protocol was achieved by extracting the protein from inclusion bodies. SCRΔ was expressed in *E. coli* BL21-Codon Plus and protein overexpression was induced with 1 mM IPTG for 12 h at 16 °C. Cells were harvested by centrifugation (6,000×*g*, 10 min, 4° C). The cell pellet was resuspended in "lysis buffer" (200 mM NaCl, 1 mM DTT, 1 mM EDTA, 20 mM Tris, pH 7.5) and treated with lysozyme (0.5 mg/ml). Next the samples were sonicated (25 cycles, 10 s on +20 s off) in a Soniprep 150 MSE probe sonicator. The suspensions were then centrifuged at 30,000×*g* (30 min, 4 °C), and the inclusion bodies, recovered in the pellet, were washed with "wash buffer" (50 mM NaCl, 1 % (v/v) Triton X-100, 20 mM Tris, pH 7.5) for two times and then another two washes with the same buffer without detergent in order to remove Triton X-100.

Finally, 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 at 30,000×*g* (30 min, 4 °C) to collect the soluble fraction. The sample in TU buffer was then applied to a HisTrap HP column. The protein was eluted in a stepwise 0–500 mM imidazole gradient in the same TU buffer. The purified protein was identified in SDS-PAGE gels and stored at −80 °C.

MBP-SCR was expressed in *E. coli* C41 and protein overexpression was induced with 1 mM IPTG for 12 h at 16 °C. The fusion protein was first purified using an amylose resin as described previously by Zhou et al. (1997). Then the protein was diluted five times 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 0–150 mM NaCl gradient in the same buffer.

The purified MBP-SCR was digested with Factor Xa (1:100) (w/w ratio) as described previously by Zhou et al. (1997, 1998). The digestion mix was diluted ten times with 150 mM NaCl, 0.1 mM TCEP, 5 mM Zwittergent 3–12, 20 mM Tris, pH 7.5, and loaded onto a HisTrap HP column equilibrated in the same buffer. Finally SCR was eluted with 10 mM imidazole in the same buffer. The purified protein was identified in SDS-PAGE gels and stored at –80 °C in 150 mM NaCl, 0.1 mM TCEP, 5 mM Zwittergent 3–12, 20 mM Tris, pH 7.5.

Protein reconstitution in vesicles

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, in the case of MBP-SCR, digested with Factor Xa at a 1:100 (w/w) for 6 h at 20 °C to remove MBP, and samples were next dialyzed against D_2O buffer in Slide-A-Lyzer Mini Dialysis units. Samples were next 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 aggregated or adhered to the tube walls.

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, followed by 1 h incubation with anti-scramblase

antibody (1:1,000). The blot was washed several times with PBS, pH 7.4, and incubated for 1 h with an HRP-linked anti-mouse antibody (1:2,000). 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).

A sucrose gradient ultracentrifugation was carried out when a higher quantity of sample was required, e.g. flip-flop assays. Briefly, 500 µl each of sample (at a 1:500 protein-to-lipid mol ratio) was overlaid with 500 µl each of the following sucrose solutions (w/v): 60, 20, 10, 5, and 2.5 %. Fractions were ultracentrifuged at 100,000×*g* for 3 h at 4 °C, six 500 µl aliquots were recovered and lipid and protein quantified.

Lipid translocation assay with pyrene-labeled phospholipids

Hundred micromolar proteoliposomes were transferred to a stirred fluorescence cuvette at 37 °C and 5 mol% of either py-PC or py-SM were added to label externally the vesicles. For py-SM experiments, initial fluorescence was recorded and 5 mM calcium was added. Fluorescence was monitored for 80 min (λ_{ex} = 344 nm, λ_{em} = 380–520 nm) on an Aminco Bowman Series 2 spectrofluorimeter, and the decay of the I_{ex}/I_{mon} ratio was attributed to calcium-induced change of the py-SM position from the outer to the inner monolayer promoted by the reconstituted proteins (Müller et al. 2000).

Intrinsic fluorescence measurements

The intrinsic fluorescence of MBP-SCR and SCRΔ was used to detect conformational changes promoted by calcium binding. The proteins were dialyzed, when required, against TC buffer (150 mM NaCl, 20 mM Tris–HCl pH 7.5) or TC +6 M urea buffer in the presence of 1 mM EDTA at 4 °C, overnight, and then against EDTA-free buffers, at 4 °C, for 6 h. Fluorescence was measured in a stopped-flow device from Bio-Logic (France) at 20 °C. The excitation wavelength was λ_{ex} = 295 nm and emission was collected through the cut off filter, λ_{em} >320 nm. Protein concentrations were 1.1 and 0.5 µM for MBP-SCR and SCRΔ, respectively.

ANS binding to MBP-SCR and SCRΔ

Binding of the fluorescent probe 1,8-ANS (1-anilino-naphthalene-8-sulfonate) was used to detect hydrophobic regions on the protein surface. Protein samples were diluted to 0.4 µM, in TC buffer in the absence or presence of calcium. Then small aliquots of an ANS solution were successively added. After each addition, the mixture was incubated with continuous stirring for 5 min (sufficient time to reach equilibrium). The fluorescence emission spectra were recorded with λ_{ex} = 360 nm and λ_{em} = 430–600 nm at 20 °C and the measurements were corrected using a blank without protein.

The calcium titration experiments were performed with 0.4 µM protein in TC buffer under the same conditions as those described above. All the measurements were corrected using a blank without protein. ANS concentration was 55 µM.

Infrared spectroscopy measurements

SCRΔ was dialyzed against TC buffer+1 mM EDTA at 4 °C overnight, in the presence of 5 mM Zwittergent 3–12 when required. The protein solution was then concentrated to 2 mg ml^{-1} and next dialyzed against TC buffer in the presence and absence of 5 mM Zwittergent 3–12 at 4 °C for 6 h in order to remove the urea and EDTA.

SCR was dialyzed against TC-5 mM Zwittergent 3–12 buffer+1 mM EDTA and then against TC-5 mM Zwittergent 3–12. Next the protein was concentrated to 2 mg ml^{-1}. The proteins were centrifuged at 25,000×*g* for 40 min at 4 °C to avoid protein aggregates and then protein aliquots (0.1 ml) were freeze-dried. Finally they were resuspended in the appropriate volume of D_2O-based buffer, in the presence and absence of calcium, and the infrared spectra were measured. The spectra were recorded in a Bruker Tensor 27 (Bruker Optik GmbH, Ettlingen, Deutschland) spectrometer equipped with a liquid nitrogen-refrigerated mercury-cadmium-telluride detector. Samples were measured using a demountable Peltier liquid cell (Biotools Inc., USA) with excavated calcium fluoride BioCell windows (BioTools), and 25 µm optical path. A total of 143 interferograms min^{-1} were generated at 2 cm^{-1} resolution and averaged over one minute intervals. Opus 5.0 software from Bruker Optics was used for data acquisition. The samples were heated from 4–80 °C at a rate of 1 °C min^{-1}.

Trypsin digestion of the reconstituted proteins

Sucrose gradient formation and ultracentrifugation were carried out as in (Yethon et al. 2003). A fraction of the reconstituted 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 0.3 M sucrose. The gradient was centrifuged at 400,000 g for 3 h, and four 250 µl fractions were collected. A dot blot was performed to check proteoliposome isolation.

Tryptic digestion of the reconstituted proteins was carried out at 37 °C. After 15 min incubation in the absence/presence of 5 mM calcium, proteolysis was initiated by addition of trypsin to proteoliposomes at a 1:50 ratio (w/w). Samples were taken at different time intervals, and the reaction was stopped by heating the samples for 5 min at 100 °C in the presence of SDS and mercaptoethanol. Then SDS-PAGE and Western Blot were performed to visualize and quantify the tryptic fragments.

Turbidimetry

The aggregation of SCR or SCRΔ was determined by measuring the apparent absorbance at 400 nm caused by increased turbidity. Protein samples in Zwittergent 3–12 were diluted to 0.4 μM, in TC buffer containing 5 mM Zwittergent 3–12 in the absence (control) or presence of 5 mM calcium in the case of SCR, or 25 mM in the case of SCRΔ. Aggregation was followed in a Cary spectrophotometer equipped with a thermostated 12-holder accessory. The temperature of the samples was 25 °C; final protein concentration in the cuvette was 0.4 μM and time-courses were recorded for 15 min.

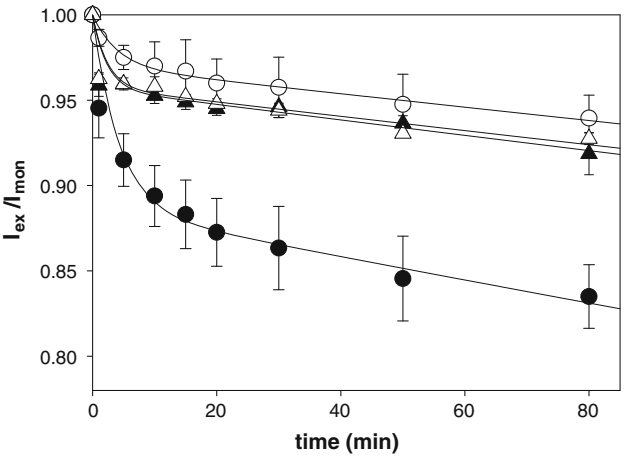

Fig. 2 Influence of SCR1 and SCRΔ proteins on py-SM transbilayer movement in PC:PS (9:1 mol ratio) LUVs (final concentration 100 μM). All measurements were made in the presence of 5 mM calcium. (*open triangle*) Liposomes. (*filled triangle*) SCRΔ proteoliposomes. (*filled circle*) SCR proteoliposomes. (*open circle*) SCR proteoliposomes in the absence of Ca^{2+}. Average values ± SEM. (n = 3–4). All curves were fitted to double exponentials of the form $y = ae^{-bx} + ce^{-dx}$. For the curve corresponding to SCR proteoliposomes, the values of the coefficients were $a = 0.113$, $b = 0.233$, $c = 0.8865$, and $d = 0.0008$. The correlation coefficient for this curve was $r^2 = 0.880$

Results

Protein purification

A novel method for the expression and purification of SCR, either native or as a fusion protein with MBP, has been developed, as described under Methods Section. The deletion mutant SCRΔ is a 32 kDa protein lacking the 28 terminal amino acids of SCR. The missing region comprises the putative transmembrane helix (residues 291–309) and the short C-terminal peptide (residues 310–318). SCRΔ has also been purified as described in the Methods Section. Gels corresponding to these three proteins can be seen in Fig. S1.

Flip-flop (scramblase) activity

The scramblase activities of SCR and of SCRΔ were assayed under similar conditions using the pyrene excimer method (Müller et al. 2000). Freshly synthesized pyrene-sphingomyelin (py-SM) was used. The geometry and structure of SM do not make it an easy lipid to undergo spontaneous transmembrane motion. The data in Fig. 2 show that in the presence of SCR, reconstituted at a 1:43 protein:lipid mol ratio, in vesicles consisting of PC:PS (9:1 mol ratio), the proportion of pyrene excimers decreases, particularly in the first 20 min after Ca^{2+} addition (5 mM Ca^{2+} is used to trigger the reaction). This is taken as an indication of transmembrane lipid motion, or flip-flop. According to the procedure mentioned under Methods Section, about 5 % of py-SM has undergone transmembrane motion in the first 20 min, a figure in agreement with data for this protein by Sims and coworkers (1998; Zhou et al. 1998) using a different methodology. Flip-flop data for SCR and Ca^{2+} can be fitted to a double exponential, that may correspond to a fast and a slow process. We suggest that the fast event corresponds to the initial outside-to-inside motion of py-SM caused by the protein when first reaching the bilayer, and that this is followed by a slower bidirectional motion of the probe once the outer monolayer has been relatively depleted of py-SM.

Under the same conditions (Fig. 2), SCRΔ (reconstituted at a 1:120 protein:lipid mol ratio) did not support any measurable flip-flop motion. Thus the C-terminal region of SCR appears to be essential for the scramblase activity. Similar results were obtained with a py-PC (Fig. S2).

Calcium binding

Binding of Ca^{2+} ions to the native and mutant proteins was assayed through changes in the Trp intrinsic fluorescence using a stopped-flow fluorescence spectrophotometer. SCR was studied as a fusion protein with MBP. The MBP fragment stabilizes SCR in aqueous media, and does not contribute to the observed fluorescence changes. Millimolar concentrations of Ca^{2+} decreased the intrinsic fluorescence intensity (Fig. 3a, b), suggesting that the overall

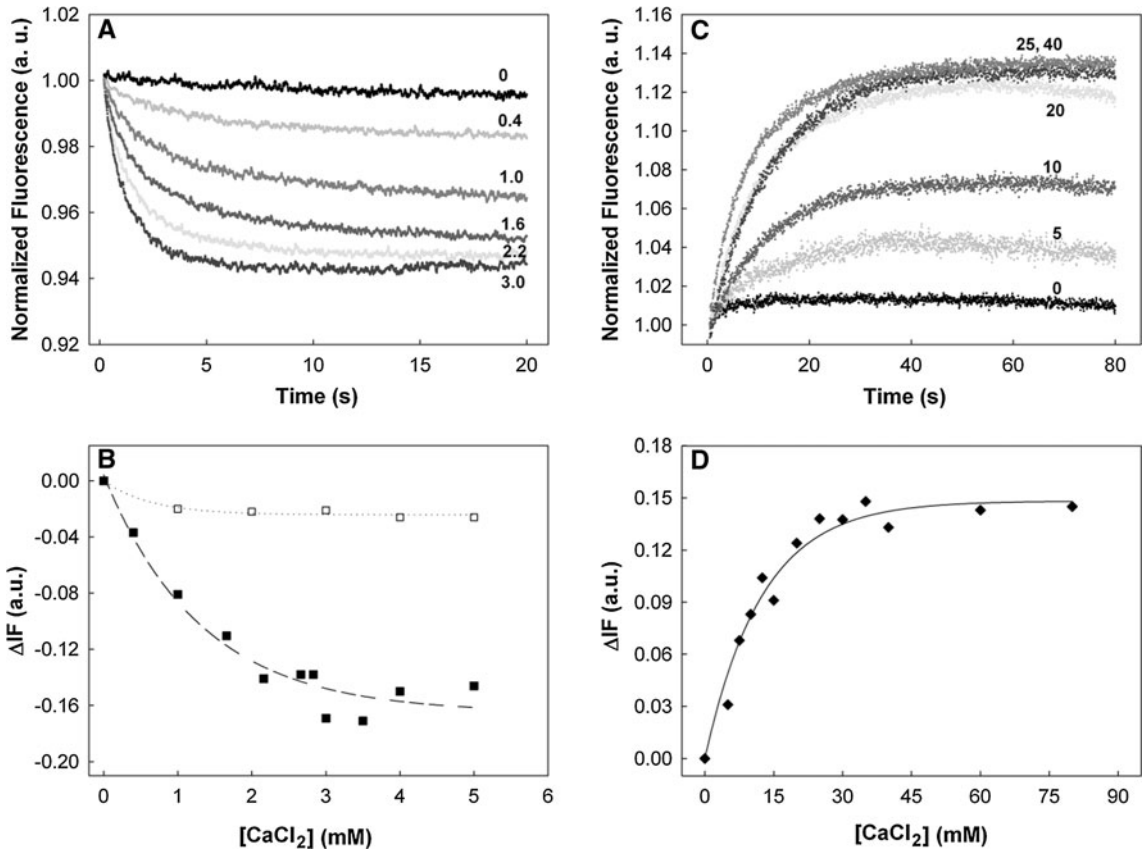

Fig. 3 Intrinsic fluorescence of (*filled square*) MBP-SCR (*open square*) MBP and (*filled diamond*) SCRΔ as a function of calcium concentration. **a**, **c** stopped-flow traces. The figures indicate the Ca^{2+} concentration. **b**, **d** change in fluorescence intensity. Protein concentration was 1.1 μM for MBP and MBP-SCR, and 0.5 μM for SCRΔ. In (**b**), (**d**), the data were fitted to hyperbolic curves. Correlation coefficients were $r^2 = 0.957$ (**b**) and $r^2 = 0.968$ (**d**)

environment of the fluorophores became more polar. The apparent Ca^{2+} binding constant was K $\sim$1 mM^{-1}. The effect of Ca^{2+} on SCRΔ was very different (Fig. 3c, d), Ca^{2+} caused an increase in intrinsic fluorescence, suggesting that in the presence of the divalent cations the Trp residues met a less polar environment. Moreover, the apparent affinity for Ca^{2+} decreased by an order of magnitude so that K $\sim$8 mM^{-1}. The Ca^{2+} binding data strongly support the notion that the structure/function of the SCR calcium-binding site (Fig. 1) is influenced by the adjacent α-helical domain, that has been deleted in SCRΔ.

Calcium structural effects

The structural effects of Ca^{2+} ions on SCR and on SCRΔ were further studied using ANS fluorescence and trypsin digestion. ANS is a commonly used fluorescent probe (Slavík 1982) whose emission intensity increases when transferred from aqueous to non-polar environments. In particular it binds non-polar regions of proteins and the binding is detected through an increased fluorescence emission. When SCR is titrated with ANS (Fig. 4a) the probe fluorescence intensity increases hyperbolically both in the presence and in the absence of Ca^{2+}, however, the increase in fluorescence intensity is about 3.5 times larger in the presence of the cation. The corresponding apparent affinity constants, however, do not show large changes, K^- $\sim$22 μM in the absence of Ca^{2+} and K^+ $\sim$18 μM in the presence of Ca^{2+}. The effect of Ca^{2+} is dose-dependent (Fig. 4b), with a half-maximal effect at $\sim$0.8 mM, in agreement with the results in Fig. 3b. In a parallel series of experiments SCRΔ was titrated with ANS (Fig. 4c, d). The results were rather similar to those with the full protein, except that the binding constants were lower for the truncated mutant, respectively, and $\sim$7 and $\sim$6 μM for the protein in the absence and presence of Ca^{2+} respectively. A major difference, however, was the Ca^{2+} concentration required to obtain half-maximal ANS binding (Fig. 4d), which was $\sim$8 mM, one order of magnitude higher than the corresponding value for SCR (Fig. 4b), and in good agreement with the results in Fig. 3d. The observations in Fig. 4 confirm the decreased affinity for Ca^{2+} of SCRΔ as compared to the native protein. The data also indicate that Ca^{2+} is increasing the number of hydrophobic regions of

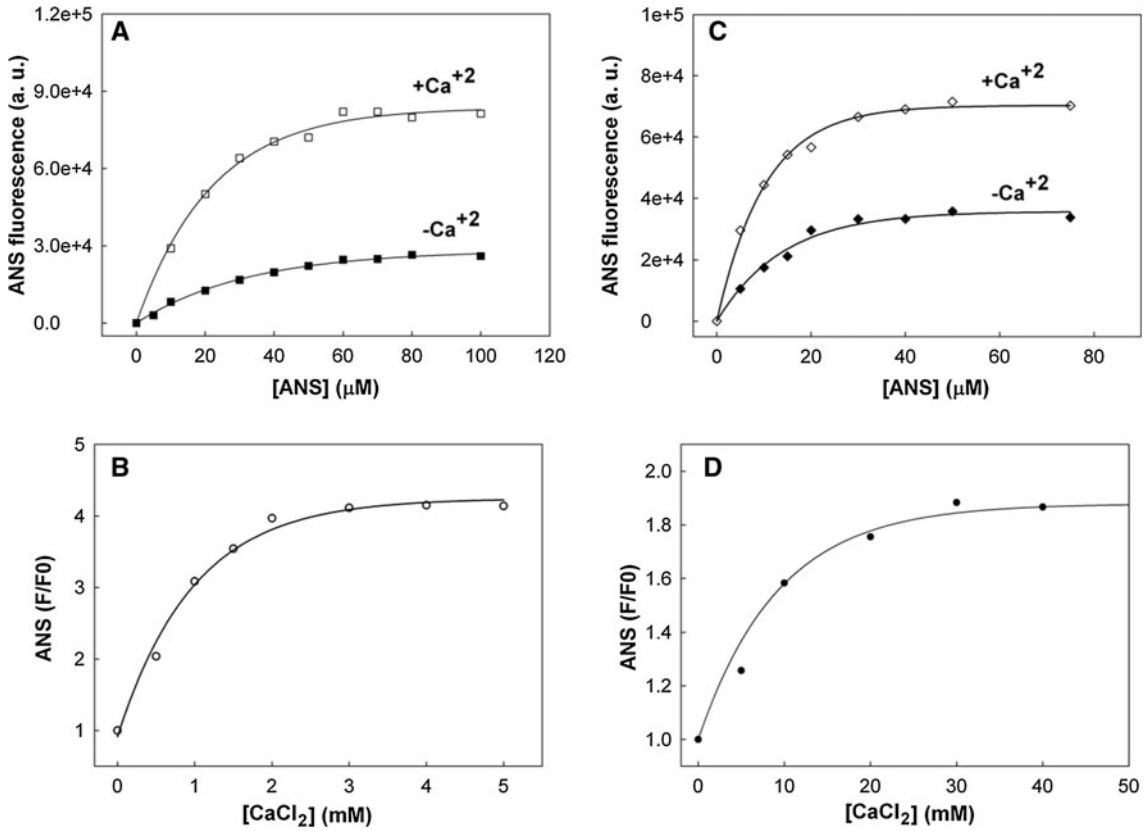

Fig. 4 **a**, **c** ANS titration of (*filled square*) MBP-SCR and (*filled diamond*) SCRΔ in the presence or absence of calcium. The protein (0.4 μM) was titrated with increasing concentrations of ANS, and ANS fluorescence emission was recorded with $\lambda_{ex} = 360$ nm and $\lambda_{em} = 430$–600 nm, in the presence and absence of 5 and 25 mM Ca^{2+} for MBP-SCR and SCRΔ, respectively. (**b**, **d**). Protein-bound ANS fluorescence intensity as a function of Ca^{2+} concentration. ANS fluorescence emission in the presence of 0.4 μM protein, and increasing Ca^{2+} concentrations, ANS concentration was 55 μM. All the measurements were corrected using a blank without protein. The data were fitted to hyperbolic curves. Correlation coefficients were: (**a**) $r^2 = 0.996$ and 0.994 in the absence and presence of Ca^{2+}, respectively; (**b**) $r^2 = 0.991$; (**c**) $r^2 = 0.985$ and 0.995 in the presence and absence of Ca^{2+}, respectively; (**d**) $r^2 = 0.987$

the protein that are accessible to the solvent, i.e., Ca^{2+} is inducing overall conformational changes in the protein.

In agreement with the latter observation, light-scattering measurements show that the spontaneous tendency of both the native protein and SCRΔ to aggregate is increased in the presence of Ca^{2+} (Fig. 5), particularly in the case of the truncated form (Fig. 5b). This is in agreement with the higher affinity of SCRΔ for ANS (Fig. 4a, c).

In order to explore whether the differential affinity for Ca^{2+} of SCR and SCRΔ was also found in the membrane-reconstituted form of these proteins both SCR and SCRΔ proteoliposomes were subjected to trypsin treatment in the absence/presence of 5 mM calcium. Depending on the tertiary structure of the protein, trypsin could have a different accessibility, rendering different trypsinisation patterns. Both SCR and SCRΔ proteoliposomes were clearly separated from the non-reconstituted protein (Fig. 6a, b). In the case of SCR proteoliposomes (Fig. 6c, e), the binding of calcium confers the protein resistance to proteolysis in comparison to the inactive protein, due to the change in the overall structure of the protein. In contrast, SCRΔ (Fig. 6d, f) is similarly digested in the absence/presence of calcium 5 mM. These results suggest that 5 mM calcium causes an important tertiary structure change in the reconstituted native protein (SCR) but not in the reconstituted truncated mutant.

Thermal stability

Thermal stability of native SCR and SCRΔ was comparatively studied, in the presence and absence of Ca^{2+}, using IR spectroscopy. Detergent (5 mM Zwittergent 3–12) was required to keep the native scramblase stable along the temperature run, and the same detergent concentration was added to SCRΔ in order to study both proteins under comparable conditions. The detergent did not cause major changes in protein secondary structure (data not shown). The IR spectrum of proteins contains the so-called amide I

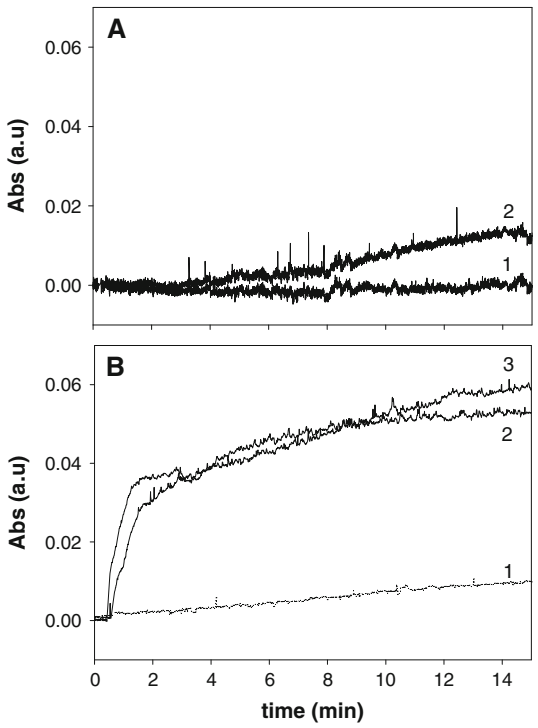

Fig. 5 Aggregation of SCR (**a**) and SCRΔ (**b**). Protein aggregation was assayed as an increase in turbidity (A_{400}). Proteins (0.4 μM final concentration) were in the presence of 5 mM Zwittergent 3–12. **a** *1* and *2* correspond to SCR in the absence and presence of 5 mM Ca^{2+}. **b** *1* and *2* correspond to SCRΔ in the absence and presence of 25 mM Ca^{2+}, respectively. *3* corresponds to SCRΔ incubated with detergent as above, then diluted in a detergent-free cuvette, in the presence of 25 mM Ca^{2+}

band, at ~1,600–1,700 cm^{-1}, arising mainly from stretching vibrations of the amide carbonyl groups in the protein backbone (Arrondo and Goñi 1999). The width-at-half-height of the amide I band tends to increase with temperature, and can be used as a marker for protein thermal denaturation. Fig. 7a, b displays amide I bands from SCR and SCRΔ at low and high temperatures, showing the increased widths after heating. Widths-at-half-height are plotted as a function of temperature in Fig. 7c, d. The amide I band of SCR (Fig. 7c) increases gradually with temperature, but the change becomes steeper respectively above ~60 and ~48 °C in the absence and presence of Ca^{2+}. This change in slope is usually taken as the onset temperature for protein denaturation. Moreover, the amide I band is about 3 cm^{-1} wider, at all temperatures, in the absence of the divalent cation. SCRΔ amide I band (Fig. 7d) increases its width with temperature in a steep way starting from the lower temperatures, with an even steeper region that occurs at ~50 °C in the absence and at ~75 °C in the presence of Ca^{2+}. Thus according to IR data, the lack of the C-terminal portion appears to make SCRΔ less thermally stable, particularly at the lower temperatures.

Discussion

The main aim of this paper was to understand the relationship of the C terminus α-helical domain of SCR with the catalysis of transmembrane (flip-flop) phospholipid motion. Secondarily, the effects of Ca^{2+} on the native protein and on a truncated mutant devoid of the C-terminal domain have also been examined.

Effects of deleting the C-terminal domain

When the last 28 amino acids of SCR, comprising the putative transmembrane α-helical domain and the C terminus peptide, are deleted, a number of important structural and functional consequences follow. First, the flip-flop catalytic activity is lost (Fig. 2, S2) thus confirming that the scramblase effect requires the presence of the C-terminal α-helix, as predicted (Zhou et al. 1997). Our results also confirm recent experimental data by Francis et al. (2013) on the role of the C-terminal α-helical domain of SCR. The precise mechanism by which SCR catalyses lipid transbilayer motion is not known, neither for this nor for other related proteins. However, a recent contribution from our laboratory (Posada et al. 2013) has shown that the C-terminal domain of SCR is capable of becoming inserted in lipid bilayers and that the exoplasmic tail contributes charged residues that can help in anchoring the protein to the bilayer.

An additional important effect of the C-terminal domain deletion is the large decrease, by one order of magnitude, of the protein affinity for Ca^{2+} (Fig. 3). The Ca^{2+} binding domain (Fig. 1, aa 273–284) remains intact in the truncated mutant, but the adjacent α-helix appears to be essential for the proper folding of the Ca^{2+}-binding site, so that helix deletion leads to poor Ca^{2+}-binding. The apparent binding constant for SCRΔ is K ~8 mM^{-1}, meaning in practical terms that under physiological conditions SCRΔ does not bind Ca^{2+}.

Binding of ANS reflects the extension of hydrophobic areas on the protein surface (Slavík 1982). In the absence of Ca^{2+} the apparent affinity of SCRΔ for ANS is about threefold higher than that of the native protein (Fig. 4a, c), and the maximum ANS fluorescence is also higher, by about 30 %, in the case of the truncated protein. This is probably also a consequence of the incorrect folding of the C-terminal domain of SCRΔ, which allows the exposure to the aqueous environment of otherwise inner hydrophobic regions. Another related effect of the C terminus is the decreased thermal stability of the mutant (Fig. 7), arguably also related to the poor folding of the truncated protein. In summary, a variety of experimental techniques concur in showing that the 28 aa at the C-terminal end of SCR, that include the putative transmembrane α-helix, constitute a

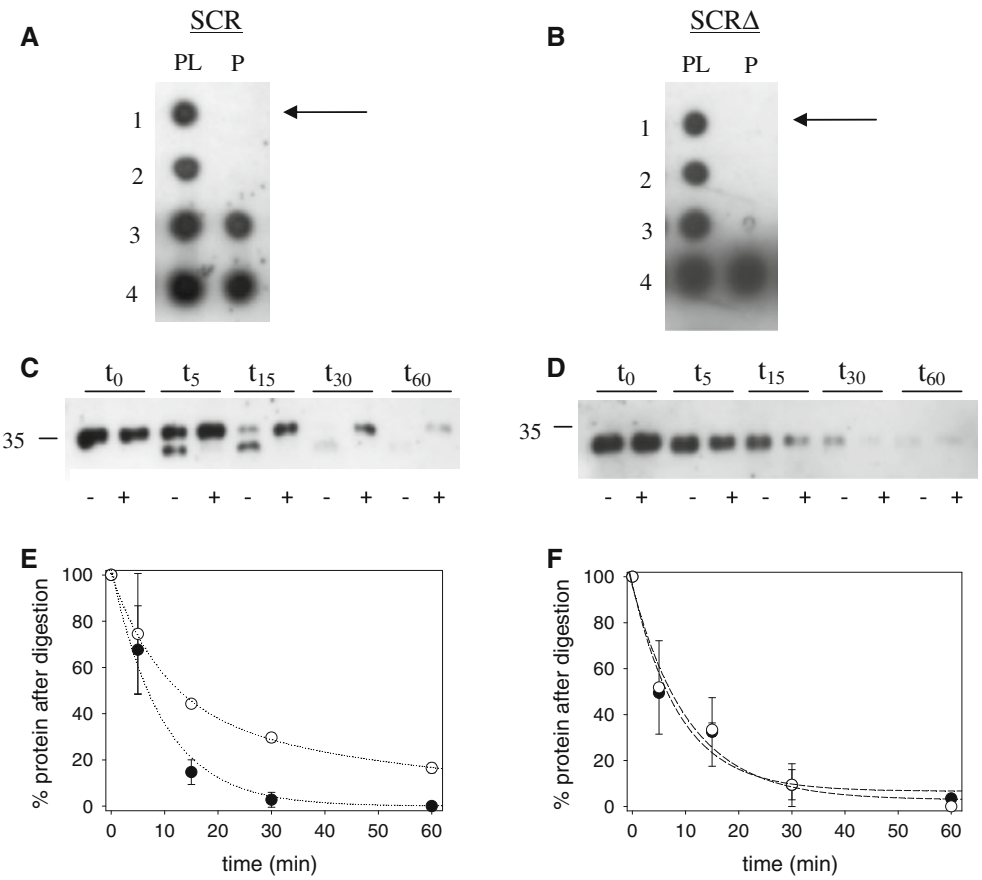

Fig. 6 (**a**, **b**) Proteoliposomes "PL" of SCR (**a**) and SCRΔ (**b**) were isolated from non-reconstituted protein by a sucrose gradient. "P" corresponds to pure protein (no reconstitution was performed). 1–4, samples recovered from top to bottom of the sucrose gradient. *Arrow* indicates the samples used in the trypsinolysis assay. (**c**, **d**) Trypsin digestion of SCR (**c**) and SCRΔ (**d**) reconstituted in liposomes. Trypsin digestion (at a 1:50 w:w trypsin:protein ratio) was carried out at 37 °C. Aliquots (in the absence/presence of 5 mM calcium) were taken at different times, indicated in minutes above *each lane*, and analyzed. A western blot was performed to visualize tryptic fragments ($n = 2$ independent experiments). (**e**, **f**) Time course of trypsin digestion of SCR (**e**) and SCRΔ (**f**) proteoliposomes in the absence (*filled circle*) and presence (*open circle*) of 5 mM calcium

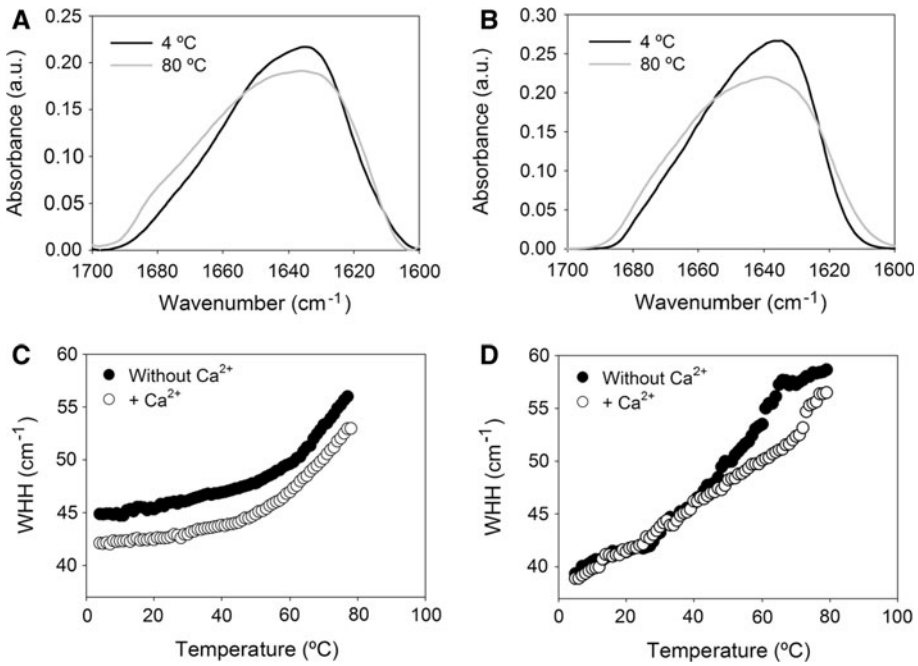

Fig. 7 Thermal stability of SCR and SCRΔ. **a**, **c** native SCR in 5 mM Zwittergent 3–12. **b**, **d** SCRΔ under the same detergent conditions. **a**, **b** Region of the protein IR spectrum corresponding to the amide I band. **c**, **d** Amide I band width-at-half-height as a function of temperature

major structural element in the proper folding and function of SCR.

Ca^{2+} effects on SCR

It is well known that SCR binds Ca^{2+} with low affinity, and that Ca^{2+} binding is essential for the scramblase activity (Bassé et al. 1996; Zhou et al. 1998; Stout et al. 1998; Sahu et al. 2009; Woon et al. 1999). Under our experimental conditions, the apparent binding constant was $\sim 1\ mM^{-1}$. In agreement with previous authors (Zhou et al. 1997) we used in these experiments a fusion protein containing maltose-binding protein (MBP) at the N terminus of SCR, in order to stabilize our protein in aqueous environments (MBP does not bind Ca^{2+}). Ca^{2+} binding to MBP-SCR was monitored through changes in the protein intrinsic fluorescence (Fig. 3). Pure MBP fluorescence does not change with Ca^{2+} (Fig. 3b). The intrinsic fluorescence intensity of the fusion protein however decreases with Ca^{2+} (Fig. 3a, b). Such a decrease is usually associated to the protein Trp residues experiencing on average a more polar environment (Bell and Biltonen 1991), i.e., Ca^{2+} would "bring out" Trp residues of SCR toward the aqueous medium. This would be in agreement with other Ca^{2+} effects on the protein, such as the increased ANS binding (Fig. 4a, b), increased protein aggregation (Fig. 5a), and decreased melting temperature (Fig. 7c). Thus we conclude that Ca^{2+} destabilizes the scramblase, and the resulting externalization of hydrophobic regions could explain an increased membrane binding of SCR, thus the Ca^{2+} requirement for phospholipid translocation. This is against the classical view that Ca^{2+} binding would stabilize proteins (Thielens et al. 1988; Chen et al. 1999; Permyakov et al. 2001), although some recent work has shown examples of Ca^{2+} binding leading to unstable protein states (Eijsink et al. 2011).

When the protein is reconstituted in lipid bilayers (Fig. 6), Ca^{2+}-binding leads to a conformational change that results in an apparent protection against trypsin hydrolysis (Fig. 6e). Such effect is not seen with reconstituted SCRΔ (Fig. 6f). This observation is related to two independent phenomena, namely the conformational change caused by 5 mM Ca^{2+} on SCR and the expected conformational change that the protein will undergo upon reconstitution in a lipid bilayer.

Ca^{2+} effects on SCRΔ

Ca^{2+} effects on SCRΔ are substantially different from those on the native protein. Moreover, they occur at non-physiological Ca^{2+} concentrations (Fig. 3d), thus their study is of experimental rather than physiological interest. In short, Ca^{2+} brings the Trp residues to an average less polar environment (Fig. 3d) and increases thermal stability above 50 °C (Fig. 7d). This overall "bundling up" and stabilizing effects are accompanied by Ca^{2+}-dependent increases in ANS binding (Fig. 4c, d) and aggregation (Fig. 5b). The latter may be due to localized effects in the defectively folded C-terminal region of SCRΔ.

Conclusions

In the current uncertain state of our knowledge on the physiological location and function of SCR in the cell (Ben-Efraim et al. 2004; Wiedmer et al. 2003; Sahu et al. 2009; Py et al. 2009; Merregaert et al. 2010), our results cannot shed light on whether the plasma membrane or the nuclear location is the physiological one, if there is a single physiological location for this protein. The data in the present paper underline the essential role of the C-terminal α-helix for flip-flop catalysis. Moreover, we have found that the C-terminal segment is essential for the overall folding of the protein, and for its Ca^{2+}-binding properties.

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