The sorcin–annexin VII calcium-dependent interaction requires the sorcin N-terminal domain

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Abstract Surface plasmon resonance experiments show that at neutral pH the stability of the complex between sorcin and annexin VII (synexin) increases dramatically between 3 and 6 μM calcium; at the latter cation concentration the K_D value is 0.63 μM. In turn, the lack of complex formation between the sorcin Ca^{2+} binding domain (33–198) and synexin maps the annexin binding site to the N-terminal region of the sorcin polypeptide chain. Annexin VII likewise employs the N-terminal domain, more specifically the first 31 amino acids, to interact with sorcin [Brownawell, A.M. and Creutz, C.E. (1997) J. Biol. Chem. 272, 22182–22190]. The interaction may involve similar structural motifs in the two proteins, namely GGYY and GYGG in sorcin and GYPP in synexin.

Key words: Annexin VII; Sorcin; Topology of interaction

1. Introduction

Sorcin (**so**luble **r**esistance related **c**alcium binding prote**in**) and annexin VII (synexin) belong to different families of calcium binding proteins that share the ability to undergo a calcium-dependent translocation from cytosol to membranes at physiological concentrations of the cation. This process is most likely related to the physiological function of the two proteins which has not been definitively established.

Sorcin belongs to the newly defined penta EF-hand family [1]. The 21.5 kDa polypeptide chain displays a two domain structure. The N-terminus (1–32), which is rich in glycine, proline and tyrosine residues, can be easily distinguished from the C-terminal domain (33–198) which contains all the EF-hand motifs [2]. Binding of Ca^{2+} to the high affinity sites EF1 and EF2 induces translocation of sorcin to membranes where it interacts with specific target proteins. This finding suggested a role of sorcin in Ca^{2+}-mediated signal transduction [3–7]. Annexin VII is likewise characterized by a two-domain organization of the polypeptide chain that is common to all members of the annexin family. The hydrophobic N-terminal domain is unusually long (167 amino acids) and contains unique GYPP motifs [8]; the C-terminal domain is formed by a four-fold repeat of the so-called endonexin fold, a conserved 70 amino acid sequence responsible for calcium and phospholipid binding [9]. Annexin VII was shown by Creutz et al. [10] to stimulate the aggregation of chromaffin granules in a Ca^{2+}-dependent manner; it is thought to be involved in the fusion processes occurring during cellular exocytosis [11] and in ion channel formation [8,11].

Recently, annexin VII was found to interact with sorcin in a Ca^{2+}-dependent manner, thereby recruiting it to chromaffin granule membranes; in turn, this interaction leads to inhibition of synexin-mediated chromaffin granule aggregation [5]. The synexin–sorcin interaction, however, is not limited to adrenal medullary tissue, but takes place also in the endoplasmic reticulum membranes of differentiating myoblasts [12].

In vitro the sorcin binding site on the annexin VII polypeptide chain was mapped to the first 31 amino acids [5]. However, it is not known which part of the sorcin chain contains the annexin VII binding site. It may be hypothesized that the N-terminal domain is involved since it contains two sequences, GGYY and GYGG, which resemble the GYPP motifs of synexin. To test this idea, we studied the ability of sorcin and of the 33–198 fragment, which corresponds to the complete sorcin Ca^{2+} binding domain (SCBD), to interact with purified annexin VII by overlay assay and surface plasmon resonance (SPR) experiments.

The SCBD fragment was chosen because the removal of the N-terminal domain does not affect the structural and functional properties of the native protein. Thus, the monomer–dimer association constant and the affinity for Ca^{2+} of SCBD are unaltered relative to native sorcin [2].

2. Materials and methods

2.1. Expression and purification of sorcin

Expression of recombinant sorcin in *Escherichia coli* BL21(DE3) cells, kindly provided by Dr. M.B. Meyers, and its purification were carried out as described [3]. The concentration of the purified protein was determined spectrophotometrically at 280 nm using the molar extinction coefficient 29 400 [3].

2.2. Expression and purification of annexin VII

The mouse annexin VII small isoform was expressed in *E. coli* using

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Abbreviations: SCBD, sorcin calcium binding domain; PVDF, polyvinylidene fluoride; TBST, 20 mM Tris–HCl, 0.5 M NaCl, 0.05% Tween 20, pH 7.5; EGTA, ethylene glycol bis(β-aminoethyl ether); HBS, 10 mM HEPES, 150 mM NaCl, 2 mM EDTA, 0.005% (v/v) surfactant P 20; EDTA, ethylenediaminetetraacetic acid; SPR, surface plasmon resonance; RU, resonance units

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pT7-7 as the expression vector as described in [12] and was purified as described in [13]. The concentration of the purified protein was determined using the method of Lowry.

2.3. Preparation of the 33–198 sorcin fragment, SCBD

The 33–198 sorcin fragment, SCBD, was obtained by limited proteolysis of the native protein with the endoproteinase AspN (sequencing grade, Boehringer Mannheim), which cleaves specifically the peptide bond N-terminal to Asp33 [2]. The reaction was carried out at 30°C for 16 h in 100 mM Tris–HCl pH 7.4 using a 0.5–1.0 mg/ml solution of sorcin and an enzyme:substrate ratio of 1:500 w/w. SCBD was separated from the undigested protein by anion exchange chromatography and its concentration was determined spectrophotometrically at 280 nm, using the molar extinction coefficient 22 000 [2].

2.4. Overlay assay experiments

Aliquots of purified annexin VII were subjected to electrophoresis on a 15% polyacrylamide gel under native conditions [14] and transferred to polyvinylidene fluoride membranes (PVDF, Problott, Applied Biosystems, Foster City, CA) in transfer buffer (25 mM Tris–HCl, 192 mM glycine, 20% methanol, pH 8.3) for 45 min at 100 mA [15]. For Western blotting analysis, the PVDF membranes were incubated at room temperature for 2 h with sorcin or with SCBD (5 μg/ml) in 1% gelatin in TBST buffer (20 mM Tris–HCl, 0.5 M NaCl, 0.05% Tween 20, pH 7.5 containing about 10^{-5} M $CaCl_2$ or 10^{-3} M EGTA). Subsequently, the membranes were incubated with anti-sorcin polyclonal antibody in 1% gelatin in TBST buffer (dilution 1:3000). The blots were developed by incubation with alkaline phosphatase conjugated monoclonal anti-rabbit IgG (Sigma, St. Louis, MO) in the same buffer (dilution 1:3000).

2.5. SPR measurements

Real time binding experiments were performed at 25°C using a BIAcoreX system (Pharmacia Biosensor, Uppsala). Annexin VII was immobilized to sensor chip CM5 (Biosense, Milan) via amine coupling. A continuous flow (5 μl/min) of HBS buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 2 mM EDTA, 0.005% [v/v] surfactant P20 in distilled water) was maintained over the sensor surface. The carboxylated dextran matrix of the sensor surface was first activated by a 7 min injection of a mixture of *N*-hydroxy-succinimide and *N*-ethyl-*N*′-(3-diethylaminopropyl)carbodiimide, followed by a 6 min injection of annexin VII ($C = 50$ μM in 20 mM sodium acetate, pH 5.0). The immobilization procedure was completed by a 7 min injection of 1 M ethanolamine hydrochloride to block remaining ester groups. The density of immobilized annexin VII ranged between 1.3 and 2.5 ng/mm^2. For control experiments, the sensor surface was treated as described above in the absence of annexin VII. Solutions of sorcin or SCBD (buffer = 10 mM HEPES pH 7.4, 150 mM NaCl, 3–6 μM $CaCl_2$, 0.005% [v/v] surfactant P20; $C = 0.12$–4.7 μM; $V = 100$ μl) were injected into the flow cell and passed over the annexin VII surface at a flow rate of 20 μl/min; their interaction was followed in real time. After each measuring cycle, the sensor surface was regenerated by injection of 40 μl of running buffer containing 2 mM EGTA to dissociate the analyte from the immobilized ligand. Sensorgrams were analyzed by non-linear least squares curve fitting using the Pharmacia BIAevaluation software [16]. The dissociation constant, K_D, of the annexin VII–sorcin interaction was determined by a Scatchard analysis from the dependence of the plateau signal at steady state on the concentration of sorcin in the mobile phase. A single site binding model ($A+B=AB$) was used for the analysis [16].

3. Results

3.1. Binding of sorcin to annexin VII

Firstly, the occurrence of interaction between native sorcin and annexin VII and its Ca^{2+} dependence were assessed by overlay assay experiments. Synexin transferred to PVDF membranes was incubated with sorcin in the presence of $CaCl_2$ or EGTA and sorcin binding was detected with polyclonal anti-sorcin antisera. Fig. 1 shows that the interaction between sorcin and annexin VII occurs in the presence of about 10^{-5} M $CaCl_2$, but not in the presence of 10^{-3} M EGTA, in accordance with the data of Brownawell and Creutz [5]. Control Western blots ruled out the existence of cross-reactivity of the anti-sorcin antibody with annexin VII.

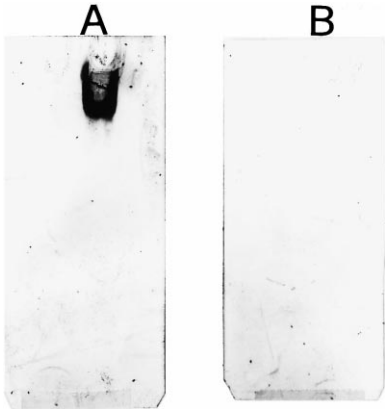

Fig. 1. Dependence on calcium of the interaction between annexin VII and sorcin. Aliquots of annexin VII (10 μg) were subjected to electrophoresis under native conditions [14] and transferred to PVDF membranes [15]. The PVDF membranes were incubated with native sorcin (5 μg/ml) in a buffer containing 10^{-5} M $CaCl_2$ (A) or 10^{-3} M EGTA (B) and subsequently treated with anti-sorcin polyclonal antibody. For buffer conditions see Section 2.

SPR was used thereafter to measure the thermodynamic parameters of the interaction and the optimal calcium concentration required. Annexin VII was immobilized on the dextran matrix of the sensor chip and a sorcin solution was passed over the surface in a running buffer containing either calcium at different concentrations or EGTA as described in Section 2. Fig. 2 emphasizes that the sensorgrams recorded in the presence of $CaCl_2$ display various phases: the initial increase in resonance units (RU) from the baseline corresponds to the association of sorcin to immobilized annexin VII during sample injection, the plateau region represents the steady-state phase of the interaction, where the rate of sorcin binding is balanced by the rate of its dissociation from the complex with immobilized annexin VII. The last phase of the sensorgram, which occurs during buffer flow at the end of sample injection, displays a decrease in RU due to the dissociation of sorcin from the complex. At 3 μM $CaCl_2$ the complex dissociates at a fast rate and the signal returns rapidly to the baseline. At 6 μM $CaCl_2$ the amplitude of the sensorgram is significantly higher and the dissociation phase is much slower than at 3 μM $CaCl_2$ indicating a significant increase in the stability of the complex. No increment of the binding signal was observed upon further increase in calcium concentration (data not shown). Fig. 2 also brings out that there is no interaction between sorcin and immobilized annexin VII in the presence of EGTA, consistent with the overlay experiments depicted in Fig. 1.

Having established that optimal interaction of sorcin with immobilized annexin VII occurs at 6 μM $CaCl_2$, the kinetic and thermodynamic constants of the interaction were determined from sensorgrams recorded at this calcium concentration as a function of sorcin concentration in the range 0.113–4.7 μM. The analysis of the data yields a K_D value of 0.63 μM (Fig. 3).

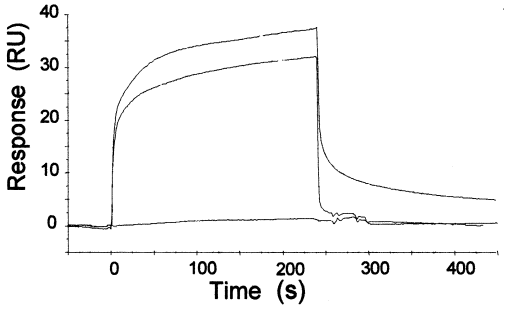

Fig. 2. Ca^{2+} dependence of sorcin binding to annexin VII. Sensorgrams of sorcin (2.3 μM) binding to immobilized annexin VII obtained in the presence of 2 mM EGTA, 3 μM $CaCl_2$ and 6 μM $CaCl_2$ (from bottom to top). The formation of the complex between sorcin and annexin VII is monitored by the increase in RU from the initial baseline; the plateau line represents the steady-state phase of the interaction, while the decrease of RU from the plateau represents the dissociation of sorcin from immobilized annexin VII.

3.2. Identification of the sorcin domain involved in the interaction with annexin VII

Synexin transferred to PVDF membranes was incubated with sorcin or SCBD in the presence of about 10^{-5} M $CaCl_2$ and complex formation was detected with polyclonal anti-sorcin antisera. The sorcin antisera employed recognized SCBD in control experiments (data not shown). Fig. 4 demonstrates that annexin VII interacts with sorcin, but not with SCBD indicating that the sorcin N-terminal domain is required for the interaction with annexin VII.

Consistent with these data, the sensorgrams recorded after injection of SCBD on annexin VII immobilized on the sensor chip surface do not provide evidence of binding in the presence of 6 μM $CaCl_2$ (Fig. 5). As expected on the basis of the data of Figs. 2 and 3, the sensorgram recorded after injection of sorcin at the same concentration of 4.7 μM indicates complex formation.

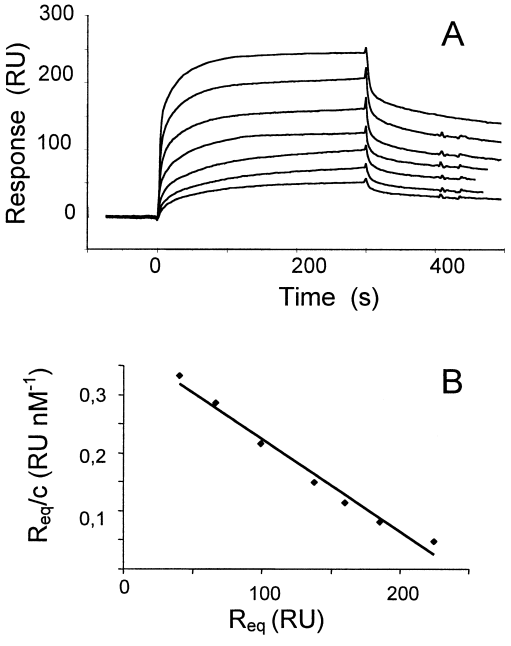

Fig. 3. Quantitative analysis of sorcin binding to annexin VII. A: Sensorgrams of sorcin binding to immobilized annexin VII carried out in the presence of 6 μM $CaCl_2$ and as a function of sorcin concentration (C = 0.12, 0.23, 0.47, 0.93, 1.4, 2.3, and 4.7 μM from bottom to top). B: Plot of R_{eq}/C versus R_{eq} at different sorcin concentrations.

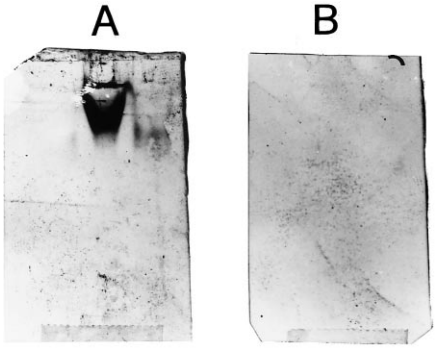

Fig. 4. Calcium-induced interaction between sorcin and SCBD with annexin VII. Aliquots of annexin VII (10 μg) were subjected to electrophoresis under native conditions [14] and transferred to PVDF membranes [15]. The PVDF membranes were incubated with native sorcin (A) or with SCBD (B) in the presence of 10^{-5} M $CaCl_2$ and subsequently treated with anti-sorcin polyclonal antibody. For buffer conditions see Section 2.

4. Discussion

The data presented in this work provide new information on the sorcin–synexin interaction that supports its physiological significance. The formation of the complex at micromolar calcium concentration and the modulation of its stability by small changes of the cation concentration between 3 and 6 μM (Fig. 2) render the interaction a potentially useful Ca^{2+}-mediated signalling mechanism in the cells where both proteins are expressed, e.g. adrenal medulla or myocytes [3,12]. In fact, this sensitivity is common to many of the signal transduction systems that control Ca^{2+}-mediated activities [17]. The strong dependence of the interaction on calcium concentration demonstrated by the present SPR data was not apparent from the experiments of Brownawell and Creutz [5]. These authors observed complex formation at 20 μM calcium, but not at 4 μM, in good agreement with the present data. No intermediate calcium concentrations were analyzed.

At 6 μM Ca^{2+} and pH 7.4 the sorcin–synexin interaction is characterized by an equilibrium dissociation constant, K_D, of 0.63 μM (Fig. 3). To our knowledge this is the first quantitative assessment of the interaction between annexins and a target protein. Brownawell and Creutz [5] observed that sorcin inhibits the annexin VII-mediated aggregation of chromaffin granules and that maximal inhibition is achieved at 2 mol of sorcin/mol of synexin. It was therefore suggested that sorcin may bind to synexin as a dimer. If one assumes that the sorcin dimer contains a single effective synexin binding site, the sensorgrams of Fig. 3 can be recalculated to yield a K_D value of 0.31 μM. Sorcin was found recently to undergo a monomer–dimer equilibrium characterized by an association constant $K_{1,2}$ of $1–3\times10^5$ M^{-1} which is not influenced by calcium binding [2]. It follows that at the concentrations used by Brownawell and Creutz (around 0.1 μM) soluble sorcin is predominantly monomeric. However, it may be envisaged that upon recruitment of sorcin at the chromaffin granule membranes higher local sorcin concentration levels can be achieved that lead to stabilization of the dimeric state.

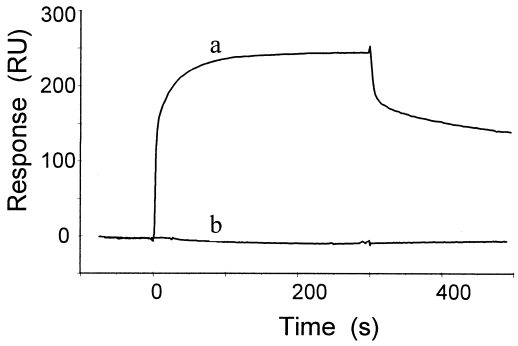

Fig. 5. SPR analysis of sorcin and SCBD binding to immobilized annexin VII. Sensorgrams obtained upon injection of sorcin (a) and SCBD (b) in the presence of 6 μM $CaCl_2$. The concentration of sorcin and SCBD was 4.6 μM.

Another important feature of the sorcin–synexin interaction brought out by the present data is the identification of the synexin binding site on the sorcin chain. The lack of interaction between synexin and SCBD, the sorcin C-terminal Ca^{2+} binding domain, depicted in Figs. 4 and 5, proves that the sorcin N-terminal domain 1–32 is required for interaction with annexin VII. This finding complements the observation by Brownawell and Creutz [5] that the N-terminal domain of annexin VII, and more specifically the very first 31 amino acids, mediate the association with sorcin. The involvement of the synexin N-terminal sequence in the interaction is not unexpected. In annexins the binding sites of target proteins are all within the N-terminal domain which is available for interaction also in the membrane-bound state [18]. The first 31 amino acids in the synexin sequence comprise several glycine residues which give rise to three GYPP structural motifs that are commonly used for protein–protein interactions [5]. Most likely these motifs interact with the GGYY and the GYGG sequences in the sorcin N-terminal domain.

Recently, it has been established that the calcium-dependent interaction between sorcin and the ryanodine receptor is mediated by the sorcin C-terminal domain [2]. Sorcin therefore can use either the N- or the C-terminal region for target protein recognition. It is, however, unknown whether sorcin can interact simultaneously with two distinct partners and thereby couple distinct intracellular signals. Differentiating myocytes, where sorcin and annexin VII are known to colocalize on the endoplasmic reticulum membrane [12] which is rich in ryanodine receptors, may serve as a model system to gain this information.

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