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Calcium-dependent translocation of sorcin to membranes: functional relevance in contractile tissue

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Abstract Sorcin, a 22 kDa calcium binding protein present in abundance in cardiac tissue and in multi-drug resistant cells and previously described as a soluble protein, is now shown to undergo a calcium-dependent translocation process from the cytosol to cellular membranes in both systems. The translocation process takes place also in *E. coli* BL21 cells that express recombinant sorcin, r-sorcin, and can be exploited in the purification of the protein. Calcium binding to purified r-sorcin occurs at micromolar concentrations of the metal and is accompanied by a conformational change that renders the protein soluble in the non-ionic detergent Triton X-114. This finding suggests that lipids are the target of sorcin on cellular membranes. The possible significance of the calcium-dependent translocation of sorcin in the specialized functions of sorcin-expressing cells is discussed.

Key words: Sorcin; Calcium-dependent membrane association

1. Introduction

Sorcin is a 22 kDa Ca^{2+} binding protein shown to be most abundant in heart by immunoblot analysis with sorcin polyclonal antibodies, but also present in kidney, brain and skeletal muscle [1,2]. The protein was originally isolated from the soluble fraction of multidrug-resistant cells, i.e. cultured cells selected for resistance to natural product cancer drugs, such as vincristine and adriamycin [3–7]. Direct calcium binding studies and in vitro phosphorylation assays have shown that sorcin purified from heart and sorcin-overproducing cultured cells binds Ca^{2+} and is phosphorylated by the protein kinase A catalytic subunit [1,5,7,8]. These findings are consistent with the presence of two EF-hand calcium binding motifs and two putative protein kinase A recognition sites in the C-terminal domain of the sorcin sequence [4]. The N-terminal domain of the sequence is rich in glycine, proline and tyrosine residues and is homologous to the corresponding domain of the calpain light chain where it is involved in heterodimer formation [4].

The role of sorcin in multidrug resistance and the biological function of the protein in normal cells have not been determined. Of interest in this regard is a recent publication about grancalcin, a protein with a high degree of sequence similarity to sorcin, identified in neutrophils and monocytes. Grancalcin displays Ca^{2+}-dependent translocation from the cytosol to the granules and plasma membrane of neutrophils, suggestive of a specialized effector role for the protein [9]. The present paper reports a similar membrane-translocation property of sorcin in both cardiac tissue and multidrug-resistant sorcin-overproducing cells. Sorcin expressed in *E. coli* with the use of a newly designed prokaryotic expression vector (pKT7SORCIN) likewise translocates to the *E. coli* membrane; the process can be used to advantage in the purification of the protein.

2. Materials and methods

2.1. Preparation of the pKT7SORCIN vector and expression of r-sorcin in E. coli

Full-length sorcin cDNA, isolated from a colchicine-resistant Chinese hamster ovary library, was a gift from Dr. Piet Borst from The Netherlands Cancer Institute [4]. It was amplified by the polymerase chain reaction (PCR) utilizing primers that contained restriction sites *Nco*I and *Hin*dIII and were synthesized by the solid-phase triester method. Oligonucleotide primers used for PCR were 5′-GGGAAAC-CATGGCGTATCCCGGGCAC-3′ (annealing to non-coding strand bases +20–38, according to the nucleotide base numbering system in [4]) and 5′-GGGAAAGGGAAGCTTTTAGACGGTCATGACACAC-3′ (annealing to coding strand bases 598–616), at the 5′ and 3′ termini of the translated region of the sorcin cDNA, respectively. The amplification product was digested with restriction enzymes *Nco*I and *Hin*dIII (Stratagene, La Jolla, CA) before ligation into the T7 promoter-based expression vector kindly provided by Dr. Barry Schweitzer [10]. The ligation product, pKT7SORCIN (Fig. 1), was used to transform competent *E. coli* BL21(DE3) cells which were plated on bacterial LB agar plates containing 0.1 mg/ml ampicillin (Boehringer-Mannheim Corp., Indianapolis, IN). Transformants were screened for the proper insert by PCR. Agarose electrophoresis was performed on the PCR product, plasmid DNA was isolated, and the sorcin cDNA sequenced before recombinant expression for verification of the clone. For growth and expression of sorcin, bacteria were inoculated into 10 ml of standard 2 × TY medium (10 g yeast extract, 16 g tryptone and 5 g NaCl per liter) containing 0.1 mg/ml ampicillin for overnight growth at 37°C, then transferred to 300 ml of 2 × TY medium with ampicillin and 5 mM $CaCl_2$. Cells were grown to mid-log phase ($A_{600\ nm} \approx 1$) before addition of isopropyl-β-D-thiogalactopyranoside (Boehringer-Mannheim Corp., Indianapolis, IN) to a final concentration of 1 mM. Sorcin was maximally expressed after induction for 30–60 min, although no indications of cytotoxicity were apparent after longer periods of growth: routinely, a 2.5 h induction period was used. Cells were harvested, washed with M9 medium [11], and frozen at −70°C or used immediately.

2.2. Purification of r-sorcin

Harvested bacteria were suspended in 10 mM Tris-HCl at pH 7.4, 10 mM NaCl, 1 mM phenylmethyl sulfonyl fluoride (PMSF), 1 mM dithiothreitol (DTT), and 0.050 mg/ml each of leupeptin, aprotinin and pepstatin, sonicated, and centrifuged at 16,000 × *g* for 5 min at 15°C.

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Abbreviations: r-sorcin, recombinant sorcin; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; EGTA, ethylene glycol bis(β-aminoethyl ether).

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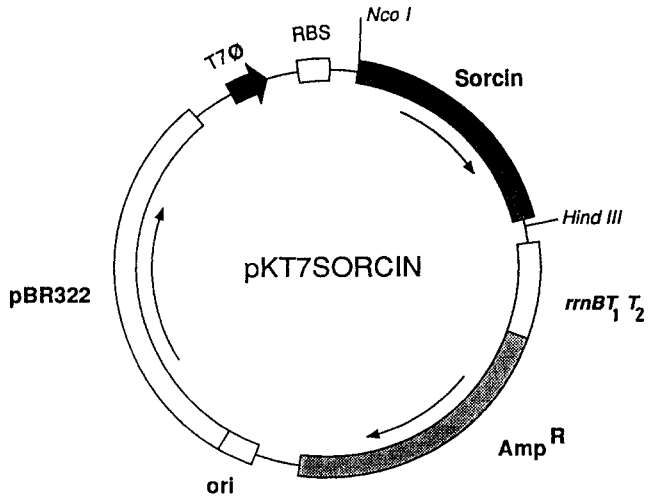

Fig. 1. Construction of sorcin prokaryotic vector, pKT7SORCIN. ori, plasmid origin of replication; Amp^R, ampicillin resistance gene; $rrnBT_1T_2$, transcript terminators; T7ø, T7 promoter.

Recovered pellets were washed repeatedly with sonication buffer; the final suspension was brought to 10 ml, was made 5 mM in $MgCl_2$ and DNase was added (0.2 μg). After 30 min incubation at room temperature, the suspension was centrifuged at 16,000 × *g* for 5 min. The recovered pellet was resuspended in sonication buffer containing 5 mM EGTA and centrifuged at 16,000 × *g* for 20 min. The supernatant of this centrifugation contains r-sorcin with nucleic acids as the main contaminants. Purification of r-sorcin was achieved by chromatography either on a MonoQ FPLC column (Pharmacia, Uppsala, Sweden) or on a DEAE-5PW HPLC column (Supelco, Bellefonte, PA) equilibrated with 10 mM Tris-HCl buffer. A linear gradient of NaCl between 0 and 0.5 M was used to recover r-sorcin which elutes at 0.25 M NaCl.

2.3. Subcellular distribution of sorcin in rabbit heart tissue and in sorcin overproducing multidrug-resistant cultured cells

Ventricular tissue of white New Zealand rabbits was minced, suspended in 10 mM HEPES buffer at pH 7.5 containing 250 mM sucrose with or without 2 mM EGTA, and lysed with a Polytron homogenizer. Suspensions were treated and centrifuged at 650 × *g*, 6,500 × *g*, 39,700 × *g* and 50,000 × *g* essentially as described in [12]. Pelleted and supernatant fractions were examined for sorcin content by Western blot analysis. Sorcin-overproducing vincristine-resistant Chinese hamster lung cells (DC-3F/VCRd-5L) [3–6] were harvested from the monolayer culture [6] and suspended in 10 mM Tris buffer at pH 7.4 containing 0.1 M NaCl, 1 mM PMSF and 1 mM dithiothreitol with 1 mM $CaCl_2$ or 2 mM EGTA. Cells were lysed with 10 stokes of a Dounce homogenizer and suspensions were centrifuged sequentially at 2,000 × *g*, 10,000 × *g* and 100,000 × *g*. Pellet and supernatant fractions were examined for sorcin content by Western blot analysis.

2.4. Gel electrophoresis and immunoblotting

Sorcin was detected by Coomassie blue staining after SDS-PAGE on 15% polyacrylamide gels [13] and by immunoblot analysis after transfer of proteins from gels to nitrocellulose [14]. The polyclonal antibody to sorcin and procedures for its use have been described [5].

2.5. Partition of r-sorcin in Triton X-114

The partition of sorcin during phase separation in a solution of the non-ionic detergent Triton X-114 was carried out as described by Bordier [15]. In brief, sorcin at 0.5–1.0 mg/ml, in 200 μl of 10 mM Tris-HCl buffer at pH 7.4 plus 150 mM NaCl and 0.6% Triton X-114, and containing either 5 mM $CaCl_2$ or 5 mM EGTA, was layered in a conical Eppendorf microcentrifuge tube on 300 μl of a solution containing 10 mM Tris-HCl buffer at pH 7.4 plus 150 mM NaCl, 0.06% Triton X-114 and 6% sucrose. The mixture was incubated for 5 min at 30°C, namely above the cloud point of the detergent. The tubes were centrifuged at 300 × *g* for 3 min at 30°C. The detergent phase, found as an oily droplet at the bottom of the tube, was clearly separated from the aqueous phase. Aliquots of the separate phases were analyzed by SDS-PAGE.

2.6. Fluorescence measurements

Intrinsic fluorescence was measured in a Jasco FP 770 fluorimeter by using excitation wavelengths of 278 and 295 nm and a slit width of 5 nm.

3. Results

3.1. Reversible Ca^{2+}-dependent association of r-sorcin with E. coli membranes

The hypothesis that sorcin, like grancalcin, associates with membranes in a Ca^{2+}-dependent fashion was tested in a model system, namely sorcin-expressing *E. coli* cells, since the highly expressed r-sorcin can be easily detected. Fig. 2A shows that

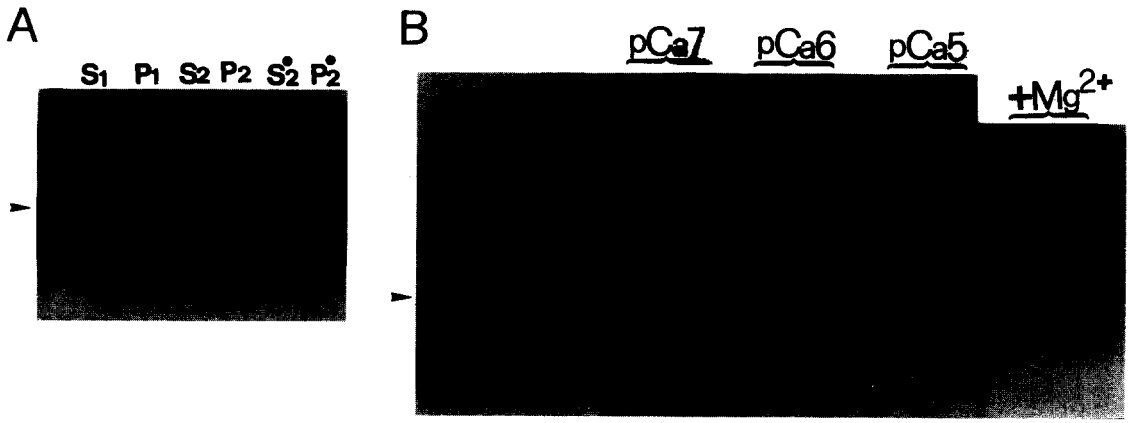

Fig. 2. Effect of Ca^{2+} and Mg^{2+} on the presence of r-sorcin in the supernatant or membrane fraction of sorcin-expressing *E. coli* BL 21 cells analyzed by SDS-PAGE. Cells lysed by sonication in 10 mM Tris-HCl, pH 7.4, containing contaminating calcium, 10 mM NaCl, 1 mM PMSF, 1 mM DTT and 50 μg each of leupeptin, aprotinin and pepstatin. (A) Cell lysate subjected to centrifugation at 16,000 × *g* for 5 min: supernatant, S_1, and pellet, P_1. P_1 resuspended twice in sonication buffer containing 5 mM EGTA and centrifuged as above yielding, respectively, S_2 and P_2 and S_2^* and P_2^*. (B) Purified sorcin and P_2 were mixed in 10 mM Tris-HCl, pH 7.0, plus 0.1 M KCl and 2 mM EGTA containing $CaCl_2$ to yield the indicated free Ca^{2+} concentrations, or in Tris buffer plus 0.2 M NaCl containing 5 mM $MgCl_2$. Even lanes, purified sorcin; uneven lanes, membrane fraction. Lanes 2 and 3, controls without added cations. The first lane on the left shows molecular weight markers (46, 30, 21.5, 14.3, 6.5 and 3.4 kDa). The arrowhead indicates sorcin. SDS-PAGE was carried out according to [13] with Coomassie blue staining.

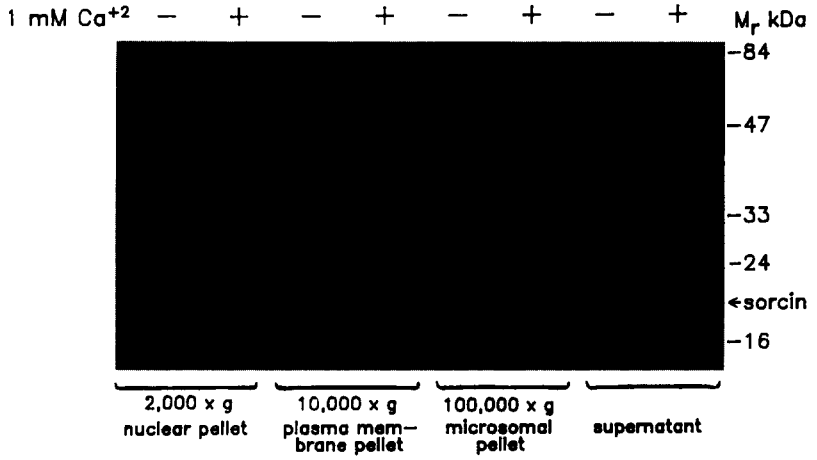

Fig. 3. Effect of Ca^{2+} on the presence of sorcin in the supernatant or membrane fractions of DC-3F/VCd-5L cells. Immunoblot analysis with polyclonal antibodies against sorcin. Homogenates of cells lysed in the presence of 1 mM $CaCl_2$ or of 2 mM EGTA, respectively, were centrifuged as described in section 2, and the pellets and supernatants analysed.

r-sorcin is associated with the membrane fraction of cells lysed in the presence of contaminating Ca^{2+} (P_1, lane 3) and that most of the sorcin is released to the supernatant upon treatment of that fraction with EGTA (S_2, lane 4; and S_2^*, lane 6). Conversely, addition of calcium to the supernatant to yield concentrations of the free cation in the micromolar range induces reassociation of the soluble protein with the membrane (Fig. 2B). The effect of Ca^{2+} is specific since addition of Mg^{2+} at millimolar concentrations does not lead to a decrease in the concentration of sorcin in the supernatant.

Having established that r-sorcin is able to translocate to *E. coli* membranes in the presence of calcium, similar experiments were performed with tissues and cells known to express natural sorcin to investigate whether the translocation process occurs with sorcin in its natural environment.

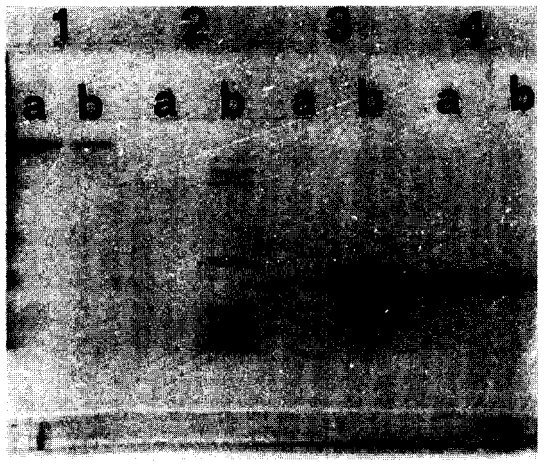

Fig. 4. Effect of Ca^{2+} on the solubility of sorcin in the aqueous or detergent phase of Triton X-114. Bovine serum albumin, cytochrome *c* oxidase and purified r-sorcin, in buffer containing 5 mM $CaCl_2$ or 5 mM EGTA, were mixed with Triton X-114 and submitted to phase separation at 30°C as described in section 2. Aliquots of the aqueous and detergent phases were analyzed by SDS-PAGE on 12% polyacrylamide gels. Lanes: 1, albumin; 2, cytochrome *c* oxidase; 3, r-sorcin in the presence of calcium; 4, r-sorcin in the presence of EGTA. Lanes a, aqueous phase; lanes b, detergent phase. Bovine serum albumin partitions in both the aqueous and detergent phase [15].

3.2. Ca^{2+}-dependent membrane association of sorcin in rabbit heart and in sorcin-overproducing multidrug-resistant cells

When heart or DC-3F/VCRd-5L sorcin-overproducing cells are lysed in the presence of EGTA, the majority of sorcin is found in the supernatant fractions, whereas when lysis takes place in the presence of calcium most of the sorcin is associated with membrane fractions (Fig. 3). In the case of rabbit heart cells, sorcin is detected principally in the $39{,}700 \times g$ and $50{,}000 \times g$ membranes which correspond to the crude and salt-extracted sarcoplasmic reticulum (SR) (data not shown).

3.3. Effect of calcium on the partition of sorcin into Triton X-114

In order to determine whether the target of sorcin during membrane association is the lipid phase or a hydrophilic site, the behavior of r-sorcin during phase separation of Triton X-114 solutions was investigated. This non-ionic detergent separates into an aqueous and a detergent phase above 20°C and thus allows one to distinguish hydrophilic from amphiphilic membrane proteins which partition differently in the two phases [15]. r-Sorcin can be recovered from the aqueous phase when phase separation takes place in the presence of 5 mM EGTA, but is found in the detergent phase when the buffer system contains 5 mM $CaCl_2$ (Fig. 4).

3.4. Ca^{2+}-dependent conformational change in r-sorcin

At a molecular level the Ca^{2+}-dependent translocation of sorcin requires a Ca^{2+}-dependent conformational change in the protein. Fig. 5 shows that incremental additions of calcium progressively quench the intrinsic fluorescence of purified r-sorcin and that the apparent K_d of calcium binding is at micromolar concentrations of the cation. As expected, based on the specificity of Ca^{2+} in inducing the translocation of sorcin, Mg^{2+} has practically no effect on the intrinsic fluorescence of the protein (upper inset to Fig. 5).

4. Discussion

Studies directed at understanding the biological role of sorcin have been based on the premise that sorcin is a cytosolic, soluble protein, as the name implies, i.e. soluble resistance-

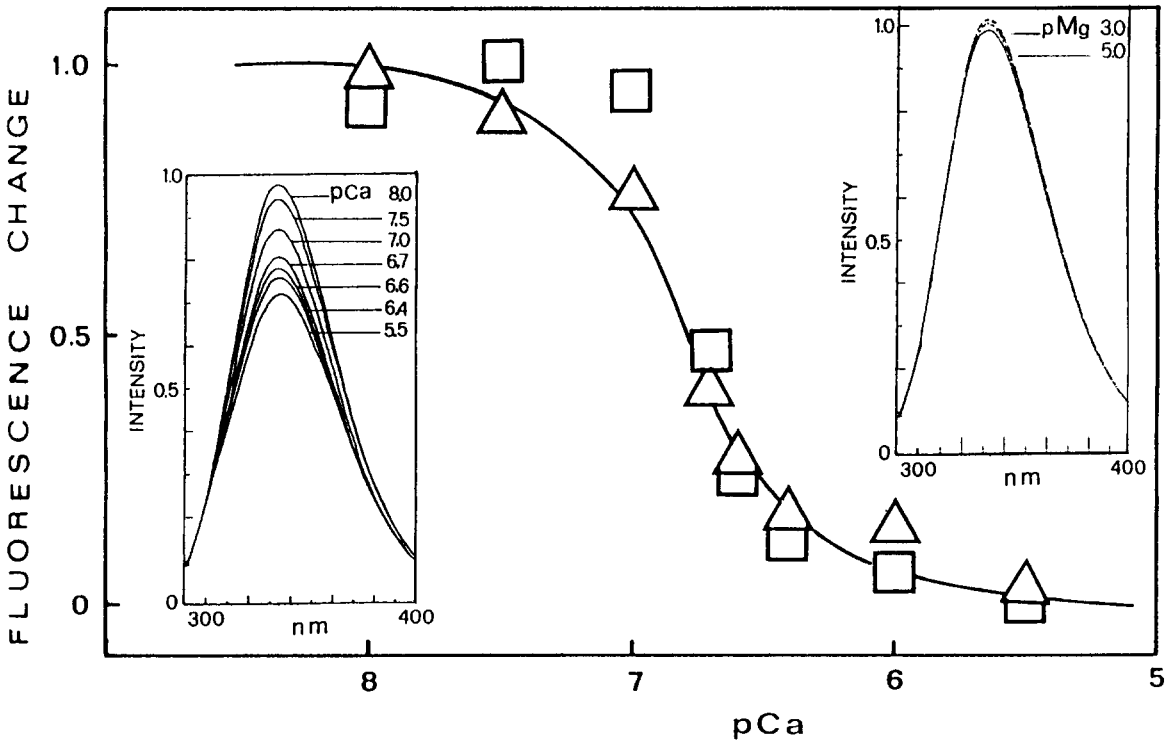

Fig. 5. Quenching of the r-sorcin intrinsic fluorescence as a function of Ca^{2+} concentration. Excitation wavelength: (△) 278 nm, (□) 295 nm; temperature, 25°C. Protein concentration, 0.2–0.3 mg/ml in 10 mM Tris-HCl containing 2 mM EGTA at pH 7.0. The insets show the fluorescence emission spectrum as a function of Ca^{2+} and Mg^{2+} concentration (excitation at 278 nm, arbitrary units). The continuous line in the upper inset shows the spectrum of sorcin in the absence of cations.

related calcium binding protein. However, the present work establishes that sorcin, when Ca^{2+}-bound, translocates to cellular and subcellular membranes, suggesting new targets for assessing sorcin's potential biological role. Ca^{2+} binding, and hence the translocation process, occur in the micromolar Ca^{2+} concentration range, which is pertinent to many signal transduction processes. For example, in the cardiac myocyte, where sorcin is expressed, the Ca^{2+} concentration changes from 10^{-7} to 10^{-5} M during the relaxation–contraction cycle, a range which brackets the K_d of Ca^{2+} binding to sorcin and suggests a potential translocation of the protein during the contractile mechanism. A further means of modulating the membrane association process of sorcin may be provided by its phosphorylation, an aspect which will be addressed in future work.

The association of sorcin with membranes is based on the occurrence of a conformational change which accompanies calcium binding. All EF-hand calcium binding proteins share the latter property [16]. However, sorcin is unusual in that intrinsic fluorescence is quenched at saturating Ca^{2+}, while in most EF-hand proteins calcium binding leads to the enhancement of fluorescence [17–19]. Calcium binding renders sorcin soluble in the non-ionic detergent Triton X-114, suggesting that the calcium-dependent association with membranes results from the interaction of sorcin with the hydrophobic lipid component. The N-terminal domain of sorcin is most likely involved in this interaction. In turn, the possible association of sorcin with a specific membrane-bound protein is rendered unlikely by the fact that such a protein should be ubiquitously distributed in markedly different membranes, like those of *E. coli* and heart cells. Based on the assumption that sorcin interacts with the lipid component, and is found in greatest abundance in the heart and in the kidney, one may speculate that it may affect ionic flux through membranes of sorcin-rich tissues. In the heart, the association of sorcin with the SR and its calcium-dependent conformational change at micromolar concentrations of the ion, together raise the possibility that sorcin might be involved in Ca^{2+} homeostasis.

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