The annexins are characterized by their ability to bind phospholipid membranes in a Ca\(^{2+}\)-dependent manner. Sequence variability between the N-terminal domains of the family members may contribute to the specific cellular function of each annexin. To identify proteins that interact with the N-terminal domain of synexin (annexin VII), a fusion protein was constructed composed of glutathione S-transferase fused to amino acids 1–145 of human synexin. Affinity chromatography using this construct identified sorcin as a Ca\(^{2+}\)-dependent synexin-binding protein. Overlay assays confirmed the interaction. The glutathione S-transferase construct associates with recombinant sorcin over the range of \(p\text{Ca}^{2+} = 4.7–3.1\) with no binding observed at \(p\text{Ca}^{2+} = 5.4\). Overlay assays using deletion constructs of the synexin N-terminal domain mapped the sorcin binding site to the N-terminal 31 amino acids of the synexin protein. Additionally, synexin forms a complex with sorcin and recruits this protein to chromaffin granule membranes in a Ca\(^{2+}\)-dependent manner. Sorcin is able to inhibit synexin-mediated chromaffin granule aggregation in a manner saturable with increasing sorcin concentrations, but does not influence the Ca\(^{2+}\) sensitivity of synexin-mediated granule aggregation. Therefore, the interaction between sorcin and synexin may serve to regulate the functions of these proteins on membrane surfaces in a Ca\(^{2+}\)-dependent manner.

The annexins are a family of homologous proteins that are characterized by their ability to bind phospholipid membranes in a Ca\(^{2+}\)-dependent manner (1). Synexin (annexin VII) was the first to be isolated (2, 3) on the basis of its ability to cause adrenal medullary chromaffin granule aggregation in vitro. Structurally, the annexins are composed of a conserved C-terminal core region containing four or eight repeats of a 70-amino acid sequence that is 40–60% conserved, and a unique N-terminal domain. The conserved core region is thought to be responsible for the annexin Ca\(^{2+}\) and lipid binding properties (4). The sequences of the N-terminal domains of this family are highly variable, leading to the hypothesis that the differences in the N-terminal domains may contribute to the specific cellular function of each annexin (9, 10, 45, 46).

Although members of the annexin family have been implicated in playing diverse roles in cellular processes that include the anti-inflammatory response (5), ion channel formation (6), vesicular trafficking (7), and exocytosis (8), the physiological function has not been definitively determined for any annexin. However, it has been proposed that the annexins may perform their in vivo roles by interacting with other proteins. In fact, several members of the annexin family have been shown to bind members of the S100 subfamily of EF-hand proteins. annexin I binds S100C (9, 10, 45), annexin II binds p11 (11), and annexin XI binds calcyclin (12) with each interaction characterized by the S100 protein binding to the N-terminal domain of the respective annexin. The annexin I-S100C and annexin XI-calcyclin interactions are Ca\(^{2+}\)-dependent, while the annexin II-p11 interaction is Ca\(^{2+}\)-independent.

Most annexins have short N-terminal domains of less than 50 residues. However, synexin (annexin VII) has an N terminal composed of 167 amino acids. This domain is largely uncharged and is rich in proline, glycine, tyrosine, and glutamine residues (13). The function of this domain is particularly enigmatic, although annexin XI also has an N-terminal domain of similar size and amino acid composition (41), and this domain has been shown to interact with calcyclin (12) and has been proposed to function as a nuclear localization signal in a cell type-specific manner (38).

The expression of mammalian annexins in yeast secretory mutants has yielded results that suggest that there may be a synexin binding protein that interacts with the N-terminal domain of the annexin. When human synexin is expressed in the late sec mutants sec2, sec4, and sec15, their growth is inhibited and their content of secretory vesicles is increased (14). Coexpression of the N-terminal domain with full-length synexin inhibits this synthetic lethality. It is possible that the effect of the N-terminal domain is due to competition with an interacting protein at a specific binding site.

Since annexins, in general, must be extracted in EGTA-containing buffers to remove them from membranes, it is possible that proteins that interact with annexins in a Ca\(^{2+}\)-dependent manner are lost during isolation of the annexins. Therefore, we have focused our attention on identifying proteins that bind annexins in a Ca\(^{2+}\)-dependent manner. To identify intracellular mammalian proteins that interact with the N-terminal domain of synexin, a fusion protein consisting of glutathione S-transferase and the N-terminal domain of synexin was constructed. Utilizing this fusion protein in affinity chromatography and overlay techniques, we have identified sorcin, an intracellular EF-hand, Ca\(^{2+}\)-binding protein, as a synexin-binding protein.

**EXPERIMENTAL PROCEDURES**

**Materials**

The following reagents were obtained from commercial sources: pGEX-KG expression vector (American Type Culture Collection, Rockville, MD) (15), glutathione and anti-glutathione S-transferase (anti-GST)\(^2\) polyclonal antiserum (Sigma), glutathione-Sepharose and CNBr-
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activated Sepharose 4B (Pharmacia Biotech Inc.), goat anti-robot HRP-conjugated antibody (HyClone Laboratories, Inc., Logan, UT), and Super Signal CL-HRP Substrate System (Pierce). Recombinant human annexin I and the synexin used in the sorcin overlays of full-length p22 was separated from the other proteins found in the peak fractions of the GST-synexin N-terminal domain fusion protein column that contained sorcin were separated by one-dimensional gel electrophoresis. These separated proteins were then transferred to nitrocellulose, and the nitrocellulose filter was blocked overnight in blocking buffer (50 mM NaCl, 8 mM NaHPO4, 2 mM NaH2PO4 (pH 7.3), 2 mM CaCl2, and 5% nonfat dry milk) at 4 °C. Individual strips were then incubated for 1 h in buffer B (20 mM Tris-HCl, 150 mM NaCl, 0.1% Tween 20, 2 mM CaCl2, and 5% bovine serum albumin) with either 5 µg/ml unfused GST protein or 5 µg/ml GST-synexin N-terminal domain construct. All wash steps consisted of three 10-min incubations with blocking buffer. The strips were washed and then incubated for 1 h in blocking buffer with rabbit polyclonal antiserum raised against GST (1:1500 dilution). Following another wash step, the strips were incubated for 1 h in blocking buffer with a goat anti-robot HRP-conjugated antibody (1:1500 dilution). Following a final wash, binding was detected using a chemiluminescent substrate system (Pierce).

Subcloning and Expression of Recombinant Human Sorcin—Sorcin cDNA was amplified by PCR from human heart cDNA (CLONTECH) using primers that incorporated Nco I and Sac I restriction sites at the 5’ and 3’ ends of the DNA, respectively. The amplified product was subcloned into pGEX-KG and used to transform E. coli XL-1 Blue. The identity of the construct was verified by DNA sequencing. Bacterial growth, protein induction, and isolation of the fusion protein were performed as described above. However, following the final wash with phosphate-buffered saline, the GSH-Sepharose beads were washed twice with cleavage buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 2.5 mM CaCl2, 0.1% β-mercaptoethanol). To cleave sorcin from the fusion protein, thrombin (Boehringer Mannheim, 0.5 unit/0.5 ml of slurry) was added to the bead slurry and incubated for 40 min at room temperature on a shaker. Released protein was recovered by washing 5–6 times with wash buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 10 mM EGTA). Recombinant protein was dialyzed overnight in 10 mM Tris, pH 7.4 with 2 mM EGTA and was further purified by application to an FPLC Poros Q anion exchange column (PerSeptive Biosystems, Cambridge, MA) equilibrated with 10 mM Tris, pH 7.4, at 4 °C and elution with a linear gradient of 0.0–1.0 M NaCl in 10 mM Tris. Sorcin eluted between 0.2 and 0.25 M NaCl. SDS-PAGE of the column fractions followed by Coomassie Blue staining of the gel revealed one major protein band at approximately 23 kDa. Polycyalon antiserum was raised in rabbits against recombinant sorcin according to methods described previously (37).

Measurement of the Ca2+ Dependence of the in Vitro Interaction of Sorcin with the GST-synexin Domain Fusion Protein—Eight micromolar concentrations of the synexin (1–145) GST fusion protein bound to GSH-Sepharose were incubated on ice for 20 min with 4 µg of recombinant sorcin in binding buffer (40 mM Heps, pH 7.0, 30 mM KCl, 240 mM sucrose, 0.1% Triton X-100) to which Ca2+–EGTA buffer was added to give a final concentration of 2.5 mM EGTA and the desired Ca2+ concentration. The final Ca2+ concentration of each buffer was verified with a calcium-selective electrode (Radiometer). After binding, the beads were rapidly washed with 5–6 times the appropriate Ca2+–EGTA binding buffer and resuspended in Laemml sample buffer. The proteins were then separated on a 10% SDS-polyacrylamide gel and were visualized by Coomassie Blue staining.

Subcloning and Expression of Recombinant Human Synexin—Full-length human synexin cDNA was amplified by PCR off the vector pCas5, which is a derivative of YEpDB80-synexin (14) in the less...
gene is disrupted. PCR was performed with primers that incorporated XhoI and Xhol restriction sites at the 5′ and 3′ ends of the DNA, respectively. The amplified product was subcloned into pGEX-KG, which was used to transform E. coli XL-1 Blue. The identity of the construct was verified by DNA sequencing. Bacterial growth, protein induction, and isolation of the fusion protein were performed as described for recombinant sorcin. However, the synexin overlay buffer consisted of 50 mM MES, pH 6.0, 150 mM NaCl, 10 mM EGTA. Recombinant synexin was dialyzed overnight in 25 mM MES, pH 6.0 with 1 mM EGTA. The protein was further purified by application to an FPLC Poros Q anion exchange column (PerSeptive Biosystems, Cambridge, MA) equilibrated with 10 mM Tris, pH 7.4, at 4 °C and elution with a linear gradient of 0–1 M NaCl in 10 mM Tris.

Construction of Deletions and Sorcin Overlay Assays—The human cDNAs for the synexin N-terminal region deletions were amplified by PCR off of the vector pGEX-KG-synexin. PCR was performed with primers that incorporated XhoI and Xhol restriction sites at the 5′ and 3′ ends of the DNA, respectively. The amplified product was subcloned into pGEX-KG and was transformed into E. coli XL-1 Blue. The identities of all of the constructs were verified by DNA sequencing. Three constructs were made that deleted C-terminal portions of the first 145 amino acids that comprise the synexin N-terminal domain. These include NT-(1–118), NT-(1–83), and NT-(1–31), where the numbering system indicates the amino acid residues of synexin that are included in the fusion protein. Five additional constructs were made that deleted N-terminal portions of this domain, and include NT-(9–145), NT-(14–145), NT-(21–145), NT-(31–145), and NT-(80–145).

Bacterial growth, protein induction, and isolation of these fusion proteins were performed as described above. GSH-Sepharose beads with 4 μg of fusion protein bound were resuspended in Laemmli sample buffer. The protein was run on a 10% SDS-polyacrylamide gel and was then transferred to nitrocellulose. The nitrocellulose filter was blocked overnight in overlay assay buffer (20 mM Tris-HCl, pH 7.4, 300 mM NaCl, 2 mM CaCl2, and 5% nonfat dry milk) at 4 °C. The immobilized proteins were then incubated for 1 h in overlay assay buffer with 2 μg/ml recombinant human sorcin. All wash steps consisted of three 10-min incubations with overlay assay buffer. The nitrocellulose filters were dried and incubated for 2 h at room temperature (2). Absorbance measurements were made using a Beckman DU70 microprocessor-controlled spectrophotometer, which allowed for absorbance data collection as a function of time for four assays simultaneously. Ca2+-EGTA buffer was added to give a final concentration of 2.5 mM EGTA, and the desired Ca2+ concentration was verified with a Ca2+ electrode (Radiometer). The initial absorbance was measured for 1 min, and then aggregation was induced by the addition of protein. Absorbance was monitored for an additional 10 min. Recombinant protein binding to granules was analyzed by pelleting the granules by centrifugation for 5 min at 14,000 × g. The pellets were resuspended in Laemmli sample buffer and run on 10% SDS-polyacrylamide gels. The supernatants were desalted by gel filtration, lyophilized, resuspended in Laemmli sample buffer, and also run on 10% SDS-polyacrylamide gels. Binding was quantitated by densitometry of the Coomassie Blue-stained gels using the Collage Image Information Program (Image Dynamics Corp.). Total protein values were calculated as the sum of the densitometric values of the protein bands on the gel and supernatant gels. For phase microscopy, chromaffin granule suspensions were mixed with both protein and CaCl2 (800 μM) and were allowed to aggregate at room temperature for 30 min before samples were imaged with a Nikon Diaphot microscope.

Analytical Methods—Protein concentrations were determined using the method of Bradford (19) using bovine serum albumin as standard. One-dimensional gel electrophoresis was performed as described by Laemmli (20) using 10% gels. The following standards were used for molecular mass determination: phosphorylase b, 97 kDa; serum albumin, 67 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 31 kDa; trypsinogen, 21 kDa; ribonuclease, 14 kDa. Gels were stained with Coomassie Blue. Western immuno blot were performed as described by Burnette (21), using horseradish peroxidase-coupled secondary antibodies and a chemiluminescent substrate system for detection (Pierce).

RESULTS

Ca2+-dependent Binding of a 22-kDa Protein to the Synexin N-terminal Domain—To determine whether cytosolic proteins of the bovine adrenal medulla were able to bind to the synexin N-terminal domain in a Ca2+-dependent manner, affinity chromatography with the GST-synexin N-terminal domain fusion protein bound to CNBr-activated Sepharose 4B was performed. The postmicrosomal supernatant from bovine adrenal medullary tissue with an added 2 mM CaCl2 was applied to the GST-synexin N-terminal domain fusion protein column. A distinct protein peak eluted from the column when Ca2+ concentration was increased from 0.2 to 2.5 mM. The protein peak was analyzed by SDS-PAGE (Fig. 1A). The protein found in the peak fraction that migrates at 22 kDa on an SDS-polyacrylamide gel appeared to bind the synexin N-terminal domain specifically because it was not observed eluting from the control column to which unfused GST was bound (Fig. 1B, c and D, compare lanes 2 and 30, respectively). The GST-synexin N-terminal domain fusion protein column was the GST control column. Several of these proteins represent protein kinase C and members of the annexin family that have been shown previously (22) to associate with the matrix, possibly through an interaction with phospholipids present in the postmicrosomal supernatant that bind nonspecifically to the column.
Identification of p22 as Sorcin—The 22-kDa protein band was excised from an SDS-polyacrylamide gel with subsequent electroelution of the protein. Following digestion of the protein with a lysyl peptidase, two of the resulting peptides were sequenced by Edman degradation. The sequences of these peptides (PFNLETCRLMVSMLDRDM and ITFDDYIACCVK) exactly match residues 69–86 and 154–165 of both hamster (23) and human (24) sorcin.

Verification of the Interaction between Sorcin and the Synexin N Terminus—Since the postmicrosomal supernatants used in the column experiments may contain small amounts of endogenous lipid, it was necessary to determine whether sorcin interacted directly with the synexin N-terminal domain or whether the association of the two proteins was lipid-dependent. Therefore, an overlay procedure was used in which the proteins from the peak fractions of the GST-synexin N-terminal domain fusion protein column were first separated by electrophoresis on an SDS-polyacrylamide gel and were then transferred to nitrocellulose. The immobilized proteins were then incubated in the presence of Ca\(^{2+}\) with either unfused GST or the GST-synexin N-terminal domain fusion protein. When binding was detected with anti-GST antiserum, the interaction between sorcin and the synexin N-terminal domain was confirmed (Fig. 2, GST-synexin N-terminal domain fusion protein). Sorcin was not able to bind to the unfused GST control (Fig. 2, GST).

Sorcin Binding to Full-length Synexin and Cytosolic Proteins of the Adrenal Medulla—To demonstrate that recombinant sorcin was able to bind full-length synexin, a sorcin overlay was performed of human synexin that had been expressed in yeast without a fusion tag. Immobilized synexin was incubated with 2 \(\mu\)g/ml recombinant human sorcin in the presence of Ca\(^{2+}\) and sorcin binding was detected with polyclonal sorcin antisera (Fig. 3A, lane 2). Additionally, sorcin overlays of total adrenal medullary cytosol demonstrated that the only protein to which sorcin bound was a 47-kDa protein, which is the expected molecular mass of synexin (Fig. 3B, lane 2). No binding of sorcin to proteins found in the total membrane fraction was observed (data not shown). Control Western blots demonstrated that background binding of the sorcin antiserum to synexin was insignificant for both experiments (Fig. 3, A and B, lane 1). Not only do these results demonstrate that sorcin binds full-length synexin, they also

**FIG. 1.** Protein concentration and SDS gels of affinity column fractions. A, protein concentration of fractions obtained from a GST synexin N-terminal domain fusion protein column. The postmicrosomal supernatant was applied to the column prior to treatment with buffers containing Ca\(^{2+}\) or EGTA as indicated at the top of the graph. Ca\(^{2+}\)-containing buffer was applied at fraction 1. The maximum of protein concentration observed at the far left of the graph represents cytosolic proteins not bound to the column. Column volume was approximately 15 ml, and the fraction size was 2.5 ml. B, 1 ml of each of the indicated fractions from the GST-synexin N-terminal domain fusion protein column was desalted, lyophilized, and run per lane on an SDS-polyacrylamide gel. The gel was then stained with Coomassie Blue. C, protein concentration of fractions obtained from a GST control column that was run as described above in A. D, 2.5 ml of each of the indicated fractions from the GST control column were treated as described in B.

**FIG. 2.** Confirmation of a direct interaction between the synexin N-terminal domain and sorcin by overlay assay. Four micrograms of the proteins in the peak fraction of the GST-synexin N-terminal domain fusion protein column that contained sorcin were separated on an SDS-polyacrylamide gel. The proteins were then transferred to nitrocellulose, and the blot was incubated with either 5 \(\mu\)g/ml GST protein only (GST) or 5 \(\mu\)g/ml GST-synexin N-terminal domain fusion protein (GST-synexin N-terminal domain fusion protein). Bound GST-protein was detected by sequentially incubating with rabbit anti-GST antiserum (Sigma), a goat anti-rabbit HRP-conjugated antibody, and a chemiluminescent substrate system (Pierce). This experiment was performed with 2 \(\mu\)M Ca\(^{2+}\) in all solutions.
Binding of Sorcin to the N-terminal Domain of Synexin

Sorcin binds full-length synexin and one 47-kDa cytosolic protein. Five micrograms of recombinant human synexin that had been expressed in yeast were run on an SDS-polyacrylamide gel and transferred to nitrocellulose. As a control for the antiserum used in the overlay, the immobilized synexin was incubated with a polyclonal antiserum raised against recombinant human synexin with any binding detected using a HRP-conjugated secondary followed by chemiluminescent detection (lane 1). An overlay assay was performed where the immobilized synexin was incubated with 2 μg/ml recombinant human sorcin with binding of sorcin detected using the same antiserum at the same dilutions as used in the control in lane 1 (lane 2). This overlay was performed with Ca2+ in all of the solutions. B. 65 μg of adrenal medullary postmicrosomal supernatant were run on an SDS-polyacrylamide gel and transferred to nitrocellulose. The control for background antiserum binding (lane 1) and the sorcin overlay assay (lane 2) were performed as described in A. Note that sorcin binds one cytosolic protein that migrates at 47 kDa, the expected molecular mass of synexin. The bands at 22 kDa represent endogenous sorcin. The faint high molecular weight bands are observed in both the control and overlay lanes.

suggest that synexin is the only soluble protein to which sorcin binds.

**Determination of the Ca2+ Requirement for Binding**—To determine the Ca2+ requirement for the in vitro interaction between sorcin and the synexin N-terminal domain, binding of recombinant sorcin to GSH-Sepharose immobilized GST-synexin N-terminal domain fusion protein was assayed at various Ca2+ concentrations (Fig. 4). The proteins were mixed for 20 min on ice in Ca2+ buffer, and then the beads were rapidly washed and loaded onto an SDS-polyacrylamide gel. Binding of sorcin to the synexin N terminus is observed over the range of pCa2+ = 4.7–3.1 (20–800 μM Ca2+) with no binding detected at pCa2+ = 5.4 (4 μM Ca2+). In control experiments using unfused GST, no binding was observed at any pCa2+ value (data not shown). Therefore, these data confirm the Ca2+ requirement for the association of these proteins and suggest that low micromolar levels of Ca2+ are required for binding.

**Mapping of the Sorcin Binding Site on Synexin**—Eight synexin deletions were constructed and expressed as GST fusion proteins. Two sets of deletions are represented, those where C-terminal portions and those where N-terminal portions of the first 145 amino acids that comprise the synexin N-terminal domain are deleted (Table I). Sorcin overlays were performed to assay the ability of sorcin to bind to each of the deletion constructs. Controls performed without any incubation with recombinant sorcin showed insignificant background binding of the sorcin antiserum (data not shown). Sorcin is able to bind the GST construct composed of amino acids 1–31 of synexin (Fig. 5B). In addition, deletion of amino acids 1–8 of synexin abolishes sorcin binding (Table I). Therefore, the results of these experiments suggest that the sorcin binding site on synexin is located at the very N terminus of the protein.

**FIG. 3.** Sorcin binds full-length synexin and one 47-kDa cytosolic protein. Five micrograms of recombinant human synexin that had been expressed in yeast were run on an SDS-polyacrylamide gel and transferred to nitrocellulose. A, as a control for the antiserum used in the overlay, the immobilized synexin was incubated with a polyclonal antiserum raised against recombinant human synexin with any binding detected using a HRP-conjugated secondary followed by chemiluminescent detection (lane 1). An overlay assay was performed where the immobilized synexin was incubated with 2 μg/ml recombinant human sorcin with binding of sorcin detected using the same antiserum at the same dilutions as used in the control in lane 1 (lane 2). This overlay was performed with Ca2+ in all of the solutions. B. 65 μg of adrenal medullary postmicrosomal supernatant were run on an SDS-polyacrylamide gel and transferred to nitrocellulose. The control for background antiserum binding (lane 1) and the sorcin overlay assay (lane 2) were performed as described in A. Note that sorcin binds one cytosolic protein that migrates at 47 kDa, the expected molecular mass of synexin. The bands at 22 kDa represent endogenous sorcin. The faint high molecular weight bands are observed in both the control and overlay lanes.

**FIG. 4.** Determination of the Ca2+ requirement for sorcin binding to amino acids 1–145 of synexin. Eight micrograms of the GST-synexin N-terminal domain fusion protein bound to GSH-Sepharose were mixed with 4 μg of recombinant sorcin in Binding Buffer for 20 min on ice. Ca2+-EGTA buffer was added to the Binding Buffer to give a final concentration of 2.5 mM EGTA and the desired Ca2+ concentration. The Ca2+ concentration of each buffer was verified with a Ca2+ selective electrode. Following a rapid wash, the proteins were subjected to SDS-PAGE and the separated proteins were visualized by Coomassie Blue staining. The pCa2+ values for each lane are listed at the bottom of the gel. The position of the sorcin band is marked by an asterisk on the gel. In control experiments using unfused GST, no binding was observed at any pCa2+ value.

**TABLE I** Sorcin binding to the synexin N-terminal domain deletion constructs

| GST construct | Sorcin binding |
|---------------|----------------|
| Full-length synexin | + |
| Syntail (amino acids 1–145) | + |
| NT-(1–118) | + |
| NT-(1–83) | + |
| NT-(1–31) | + |
| NT-(9–145) | + |
| NT-(14–145) | + |
| NT-(21–145) | + |
| NT-(31–145) | + |
| NT-(80–145) | + |

* Sorcin overlays (2 μg/ml) of the GST-synexin N-terminal domain deletion constructs were used to determine sorcin binding. All assays were performed in the presence of Ca2+. Sorcin binding was detected using a polyclonal anti-sorcin antiserum followed by a HRP-conjugated secondary and chemiluminescent detection.

**Ca2+-dependent Binding of Sorcin to the N-terminal Domain of Annexin XI**—Human synexin and annexin XI share complete sequence identity within their first eight N-terminal amino acids (MSYPGYPP). Additionally, the extreme N-terminal regions of both of these annexins contain a repeat of the sequence GYPP, although the spacing between the repeats varies between the two proteins (Fig. 10). Since deletion mapping studies suggested this region of synexin was necessary for sorcin binding, a GST-annexin XI N-terminal domain fusion protein (amino acids 1–121) was used to assay sorcin binding to annexin XI.

Binding of recombinant sorcin to the GST-annexin XI N-terminal domain fusion protein attached to GSH-Sepharose was assayed at several Ca2+ concentrations (Fig. 6). Sorcin binds the annexin XI N-terminal domain in a Ca2+-dependent manner. It appears that sorcin may bind annexin XI with lower affinity than synexin, since smaller amounts of sorcin are bound than seen with the synexin N-terminal domain (compare with Fig. 4). In control experiments using unfused GST, no binding was observed at any pCa2+ value (data not shown). These data suggest that sorcin may recognize the GYPP motif.
In control experiments using unfused GST, no binding was observed at gel. The position of the sorcin band is marked by an asterisk. This overlay was performed with all solutions containing human sorcin with binding of sorcin detected with polyclonal sorcin antisera. This overlay was performed with all solutions containing Ca\(^{2+}\). Lane 1, unfused GST; lane 2, GST-synexin (full-length); lane 3, GST-synxtail (amino acids 1–145); lane 4, GST-NT-(1–118); lane 5, GST-NT-(1–83); lane 6, GST-NT-(1–31). Control experiments demonstrated that background sorcin antiserum binding to the deletion constructs was insignificant. Note in particular the absence of an interaction of sorcin with GST alone (B, lane 1) and the strong binding of sorcin to the first 31 amino acids of synexin (B, lane 6).

**FIG. 5.** Sorcin binds amino acids 1–31 of synexin. Five micrograms of each GST fusion protein were run on an SDS-polyacrylamide gel and were transferred to nitrocellulose. A, the fusion proteins and their degradation products were visualized by Western blot with a polyclonal antiserum raised against GST (Sigma), followed by chemiluminescent detection. B, an overlay assay was performed where the immobilized fusion proteins were incubated with 2 µg/ml recombinant human sorcin with binding of sorcin detected with polyclonal sorcin antisera. Ca\(^{2+}\) antisera. This overlay was performed with all solutions containing Ca\(^{2+}\). Lane 1, unfused GST; lane 2, GST-synexin (full-length); lane 3, GST-synxtail (amino acids 1–145); lane 4, GST-NT-(1–118); lane 5, GST-NT-(1–83); lane 6, GST-NT-(1–31). Control experiments demonstrated that background sorcin antiserum binding to the deletion constructs was insignificant. Note in particular the absence of an interaction of sorcin with GST alone (B, lane 1) and the strong binding of sorcin to the first 31 amino acids of synexin (B, lane 6).

**FIG. 6.** Sorcin binds the annexin XI N-terminal domain in a Ca\(^{2+}\)-dependent manner. Eight micrograms of the GST-annexin XI N-terminal domain fusion protein bound to GSH-Sepharose were mixed with 4 µg of recombinant sorcin in Binding Buffer for 20 min on ice. Ca\(^{2+}\)-EGTA buffer was added to the Binding Buffer to give a final concentration of 2.5 mM EGTA and the desired Ca\(^{2+}\) concentration. The Ca\(^{2+}\) concentration of each buffer was verified with a Ca\(^{2+}\)-selective electrode. Following a rapid wash, the proteins were subjected to SDS-PAGE and the separated proteins were visualized by Coomassie Blue staining.

**Fig. 7.** Synexin recruits sorcin to the chromaffin granule membrane. Eight micrograms recombinant human synexin and 4 µg recombinant human sorcin were added to chromaffin granules at various Ca\(^{2+}\) concentrations, and the mixtures were incubated at room temperature for 10 min. Then the granules and any associated proteins were pelleted, the supernatants removed, the pellets resuspended in Laemmli sample buffer, and these pellets and supernatants run on separate SDS-polyacrylamide gels. A, results of the densitometry of the sorcin; B, synexin bands from the SDS-polyacrylamide gels; shaded boxes represent the percent of total protein associated with the pellet, and open boxes represent the percent of total protein found in the supernatant at the indicated pCa\(^{2+}\) value. Total protein values were calculated as the sum of the densitometric values of the protein bands on the pellet and supernatant gels. Binding assays controlling for sorcin binding to granule membranes in the presence of EGTA or at pCa\(^{2+}\) 3.1, respectively, are represented by the far right-hand lanes of A, labeled *E and *S.

A Ca\(^{2+}\)-dependent manner, the ability of both sorcin and synexin to bind chromaffin granules in the presence of Ca\(^{2+}\) was assayed. Equal molar amounts of recombinant human sorcin and synexin were added to chromaffin granules at various Ca\(^{2+}\) concentrations. Association of sorcin with granule-bound synexin was determined by densitometry of SDS-polyacrylamide gels of the granule pellets and corresponding supernatants (Fig. 7). In control assays of sorcin alone, approximately 5.9 ± 1.0% (n = 8) of sorcin is associated with the chromaffin granule pellet in the presence of EGTA (Fig. 7A, far right lane, *E). However, approximately 25.6 ± 3.2% (n = 8) of sorcin is associated with the chromaffin granule pellet at pCa\(^{2+}\) = 3.1 (800 µM Ca\(^{2+}\)) (Fig. 7A, far right lane, *S). Despite this Ca\(^{2+}\)-dependent pelleting of sorcin, which may represent either membrane binding or pelleting of the protein itself, synexin is able to recruit 90.7 ± 1.6% (n = 8) of the total sorcin to the chromaffin granule membrane surface in the presence of Ca\(^{2+}\). Sorcin remains bound to synexin and is found associated with the pellet down to approximately pCa\(^{2+}\) = 4.7 (20 µM Ca\(^{2+}\)), below which synexin is unable to bind to the granule membrane and both proteins are found in the supernatant. In control incubations with synexin alone, the amount of synexin found in both synexin and annexin XI as its binding target sequence.
bound and the Ca\(^{2+}\) dependence of synexin binding to the granules were identical to those seen in the presence of sorcin. Therefore, sorcin does not interfere with or enhance binding of synexin to the granule membrane.

Inhibition of Synexin-mediated Chromaffin Granule Aggregation by Sorcin—Since synexin is able to bring two membranes together in a Ca\(^{2+}\)-dependent manner as demonstrated by its ability to aggregate chromaffin granules (2), we wanted to determine whether the recruitment of sorcin by synexin to chromaffin granule membranes alters synexin-mediated aggregation. In particular, we wanted to determine whether sorcin is able to alter the Ca\(^{2+}\) requirement of synexin-mediated aggregation, since p11 binding to annexin II decreases the Ca\(^{2+}\) requirement for annexin II-mediated aggregation (26). Sorcin alone was found to have no chromaffin granule aggregating ability. However, addition of equal molar sorcin and synexin to the aggregation assay inhibits the aggregating ability of synexin by approximately 40% at pCa\(^{2+}\) 3.1 (800 \(\mu\)M Ca\(^{2+}\)) when measured as the percentage of the initial absorbance at 540 nm. This inhibition is saturable with increasing molar concentrations of sorcin with a maximal inhibition of approximately 70% of the initial absorbance at pCa\(^{2+}\) 3.1 observed at a 2 to 1 molar excess of sorcin (Fig. 8). Densitometry of the synexin and sorcin bands on gels of the pellets and supernatants from these experiments yielded an estimate of the stoichiometry of the interaction of 1 mol of synexin to 2–3 mol of sorcin. Although these data show that sorcin I did not recruit sorcin to the granule membrane (data not shown).

DISCUSSION

The results presented here demonstrate that the EF-hand-containing, Ca\(^{2+}\)-binding protein sorcin binds to the N-terminal domain of synexin in a Ca\(^{2+}\)-dependent manner. Sorcin stands for soluble resistance-related Ca\(^{2+}\)-binding protein. It was first identified as a protein overexpressed in conjunction with the membrane-bound drug transporter, P-glycoprotein, in multidrug-resistant cell lines selected for with natural product chemotherapeutic drugs such as colchicine or the vinca alkaloids (23). Amplification of the gene for sorcin in these cell lines,

![Phase microscopy of chromaffin granules in the presence of synexin and sorcin.](image1)

![Phase microscopy of chromaffin granules in the presence of synexin and sorcin.](image2)
FIG. 10. A conserved sequence motif is present in the synexin N terminus. Residues 1–31 of synexin contain a threefold GYPP repeat. This repeat unit is conserved between the human and mouse homologue. A glycine-, tyrosine-, and proline-rich region is also observed in Xenopus synexin and the Dictostelium homologue has 19 sequential GYPPQQ repeats. Human annexin XI also contains two GYPP repeats at the N terminus of the protein, and the first 8 amino acids of this protein are identical to those found in human synexin.

However, may be due to linkage with P-glycoprotein-encoding genes (27).

Direct binding of Ca$^{2+}$ to sorcin has been demonstrated by $^{45}$Ca$^{2+}$ overlays (28, 29), and affinity measurements have been made by studying intrinsic fluorescence changes of recombinant sorcin that occur at 0.1–1 μM Ca$^{2+}$ (25). The sequence of this protein predicts that it has four putative Ca$^{2+}$-binding domains, two with strong homology to calmodulin EF-hand motifs (23). Additionally, the N-terminal domain of sorcin is rich in glycine, proline, and tyrosine residues and is homologous to the corresponding domain of the calpain light chain. The tissue distribution of sorcin and synexin have been independently demonstrated by Western (30) and Northern blot (32) analysis to be similar. Both proteins are expressed in liver, heart, brain, lung, spleen, kidney, and, as found in our study, the adrenal medulla.

The binding sites of S100C on annexin I (10), p11 on annexin II (11), and calycin on annexin XI (31) have been determined to be within the N-terminal domain of the annexin protein. Therefore, the sorcin-synexin interaction represents the fourth example of an annexin interacting with an EF-hand-binding protein via the N-terminal domain of the annexin. Additionally, like the annexin I-S100C and annexin XI-calciyclin binding protein via the N-terminal domain of the annexin.

The Ca$^{2+}$-dependent binding of sorcin to synexin may regulate the interaction under physiological conditions. It may play a role in Ca$^{2+}$-mediated association or ion channel formation. As demonstrated with the chromaffin granule binding studies, sorcin may regulate the cardiac maffin granule binding studies, synexin may regulate sorcin by interfering with synexin self-association and may act to inhibit synexin-mediated aggregation.

The results of the overlay assays of the deletion constructs suggest that sorcin interacts specifically with the first 31 amino acids of synexin. Additionally, residues 1–8 are necessary to promote binding. It may be of importance that this exon region of synexin contains a threefold sequence repeat (GYPP). This repeat unit (Fig. 10) is conserved between the human (32) and mouse (33) homologues. A glycine-, tyrosine-, and proline-rich region is also observed in Xenopus synexin (34) and the Dictostelium homologue has 19 sequential GYPPQQ repeats (35, 36). Sequence modeling of proline repeat units has produced results that suggest these sequences form novel secondary structures composed of polyproline β-turn helices (42). In fact, the secondary structure of the annexin I and II N-terminal domains may play an important role in S100 protein binding (45). The p11 binding site on annexin II (44, 46) is predicted to form a similar helix with Met-2, Val-3, Phe-6, and Leu-7 providing potential contacts for S100C (10). These precedents suggest that the secondary structure of the synexin repeat unit may play a role in sorcin’s recognition of its binding site on synexin.

Sorcin binding to the annexin XI N-terminal domain was unexpected due to the fact that calciyclin was previously identified as a Ca$^{2+}$-dependent binding protein for annexin XI. However, it is important to note that the calcyclin binding site on annexin XI has been determined to be between amino acids 27–53 of the protein (31). Since human synexin and annexin XI share complete sequence identity within their first eight amino acids and annexin XI also contains two GYPP repeats, the binding data suggests that sorcin binds annexin XI early within its N-terminal domain. The GYPP repeat region of annexin XI ends at amino acid 15 and, therefore, the proposed sorcin binding site would not overlap with the known calcyclin binding site.

The studies with chromaffin granules demonstrated that sorcin is able to alter synexin-mediated aggregation, but not membrane binding. The control experiments with annexin I suggest that sorcin is not simply preventing the granules from making contact with each other. If this were the case, annexin I-mediated aggregation would be inhibited as well. Nor is sorcin decreasing the ability of synexin to aggregate granules simply by binding the membrane and preventing or excluding the annexin from interacting with the phospholipid membrane.

In fact, the results of the densitometry from the chromaffin granule binding studies show that the phospholipid binding ability and Ca$^{2+}$ sensitivity of synexin appears unaltered in the presence of sorcin. Therefore, the protein-protein interaction between sorcin and synexin appears to play a role in this observed inhibition of aggregation. Since the inhibition was saturated at 2 μl of sorcin/μl of synexin, sorcin may bind to synexin as a dimer. Synexin has been shown to undergo self-association in vitro in the presence of Ca$^{2+}$ (43). The self-association has the same Ca$^{2+}$ dependence and cation specificity as the aggregation of chromaffin granules by synexin. Additionally, it has been proposed that synexin self-association may play a role in synexin-mediated granule aggregation. Therefore, the binding of a sorcin dimer to synexin may interfere with synexin self-association and may act to inhibit synexin-mediated granule aggregation.

This study of the in vitro biochemical properties of the synexin-sorcin interaction may provide important tools to enhance understanding of the physiological functions of both proteins. The Ca$^{2+}$-dependent binding of sorcin to synexin may regulate synexin involvement in membrane trafficking, lipid organization, or ion channel formation. As demonstrated with the chromaffin granule binding studies, synexin may regulate sorcin by recruiting it to membranes. Recently, it has been demonstrated that sorcin associates with (30) and modulates (39) the cardiac ryanodine receptor when added to the cytoplasmic side of the receptor. In this role, sorcin, and possibly synexin by association, may play a role in Ca$^{2+}$ homeostasis in certain cell types. In the future, disruption of the synexin-sorcin interaction in...
cells using peptides or expression constructs incorporating the sequence of this binding site may provide insight into the cell biological role of this complex.

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