Recently, a human ortholog of mouse calcyclin (S100A6)-binding protein (CacyBP) called SIP (Siah-1-interacting protein) was shown to be a component of a novel ubiquitinylation pathway regulating β-catenin degradation (Matsuzawa, S., and Reed, J. C. (2001) Mol. Cell 7, 915–926). In murine brain, CacyBP/SIP is expressed at a high level, but S100A6 is expressed at a very low level. Consequently we carried out experiments to determine if CacyBP/SIP binds to other S100 proteins in this tissue. Using CacyBP/SIP affinity chromatography, we found that S100B from the brain extract binds to CacyBP/SIP in a Ca2+-dependent manner. Using a nitrocellulose overlay assay with 125I-CacyBP/SIP and CacyBP/SIP affinity chromatography, we found that this protein binds purified S100A1, S100A6, S100A12, S100B, and S100P but not S100A4, calbindin D9k, parvalbumin, and calmodulin. The interaction of S100 proteins with CacyBP/SIP occurs via its C-terminal fragment (residues 155–229). Co-immunoprecipitation of CacyBP/SIP with S100B from brain and with S100A6 from Ehrlich ascites tumor cells suggests that these interactions are physiologically relevant and that the ubiquitinylation complex involving CacyBP/SIP might be regulated by S100 proteins.

The S100 protein family consists of about 20 low molecular weight Ca2+-binding proteins (2, 3) that possess two EF-hand Ca2+-binding motifs and share about 20–80% homology in amino acid sequence. S100 proteins exhibit cell- and tissue-specific expression, and some of them bind not only Ca2+ but also Zn2+ and Cu2+ (4). Members of the S100 family bind different protein targets that modulate their activity. It has been shown that S100 proteins regulate the activity of glycogen phosphorylase (5), phosphoglucomutase (6), twinin kinase (7), nuclear Ndr kinase (8), adenylate and guanylate cyclases (9, 10), and myosin (11, 12). Several members of the S100 family bind to the cytoskeleton and regulate its polymerization and depolymerization (13). It has also been suggested that S100 proteins, through binding to annexins, might be involved in exocytosis/endocytosis processes (14). In some cases S100 proteins inhibit phosphorylation of cellular proteins by binding to the substrate. For instance S100B inhibits the phosphorylation of the tumor suppressor p53 (15).

S100A6 was originally identified in Ehrlich ascites tumor (EAT)1 cells (16, 17). It was later established that S100A6 is mainly present in epithelial cells and fibroblasts (18). We found that in EAT cells of epithelial origin, S100A6 bound a protein with a molecular mass of about 30 kDa in a Ca2+-dependent manner (19). Since this 30-kDa protein had no sequence similarity to any other known protein, we named it calcyclin-binding protein (CacyBP) (20). The region of CacyBP (residues 178–229) that binds S100A6 was then identified, and the dissociation constant of the complex was measured (21). Also, the Ca2+-dependent translocation of CacyBP/SIP within the cell was reported (22). Recently, a human protein called SIP (Siah-1-interacting protein), a component of a novel ubiquitinylation pathway regulating β-catenin degradation, was described (1). SIP shares 93% sequence identity with CacyBP, strongly implying that SIP is a human orthologue of mouse CacyBP.

We found by Northern and Western blot analysis that CacyBP/SIP is present in different rat tissues, with highest expression in the brain (23). Since a very low expression of S100A6 is observed in this tissue we designed a series of experiments to discover other S100-like protein ligands of CacyBP/SIP in the brain.

MATERIALS AND METHODS

Chemicals—Newborn calf serum, fetal bovine serum, trypsin-EDTA solution, penicillin, and streptomycin were purchased from Invitrogen; leupeptin, aprotinin, phenylmethylsulfonyl fluoride, soybean trypsin inhibitor, dithiothreitol, bovine serum albumin, monoclonal antibodies against histidine tags, monoclonal antibodies against S100A1, S100A6, and S100B, and anti-rabbit and anti-mouse IgG conjugated with horse-radish peroxidase were purchased from Sigma. Other chemicals were obtained from the companies indicated in the text.

Plasmid Construction—pET28a-CacyBP/SIP was prepared from pET30-CacyBP/SIP vector (21). The coding region of CacyBP/SIP was excised from pET30-CacyBP/SIP using NdeI and EcoRI restriction enzymes, gel purified, and ligated with pET28a vector linearized using the same restriction endonucleases. The ligation product was transformed into Escherichia coli (TOP10F†), and the presence of the insert was confirmed using the colony PCR method. pET28a-CacyBP/SIP-(155–229) was prepared from pET28a-CacyBP/SIP using inside-out PCR with Pfu polymerase. The PCR product was ligated and transformed into E. coli (TOP10F†). Constructs were verified by DNA sequencing.

Expression and Purification of Histidine-tagged CacyBP/SIP and Histidine-tagged CacyBP/SIP-(155–229)—pET28a-CacyBP/SIP or pET28a-CacyBP/SIP-(155–229) plasmid was introduced into E. coli (BL21). For protein expression, bacteria were cultured until the OD600 reached ~0.7, and IPTG was added to a final concentration of 0.4 mM. After 3 h bacteria were harvested and lysed by sonication in buffer A (50
mm NaH₂PO₄, 300 mM NaCl, 10 mM imidazole) containing protease inhibitors (Complete™, Roche Diagnostics GmbH, Germany). The lysate was cleared by centrifugation for 15 min at 27,000 × g and then applied to a Ni-NTA-agarose column equilibrated with buffer A. The column was washed with buffer A, and then the protein was eluted with a gradient of buffer A/buffer B (50 mM NaH₂PO₄, 300 mM NaCl, 100 mM imidazole). For some experiments the histidine tag was cleaved off using biotinylated thrombin (Novagen). Approximately 1 unit of the protease was used per milligram of purified histidine-tagged protein. The reaction was carried out in buffer containing 20 mM Tris-HCl, pH 8.4, and 150 mM NaCl at room temperature for 16 h. Protease was removed using streptavidin-agarose beads (Novagen).

**Other Proteins—** S100A6 was purified as described by Filipcek et al. (24). S100A1, S100B, and calmodulin were obtained from Sigma. Parvalbumin was a gift from Dr. C. W. Heizmann (Department of Pediatrics, University of Zurich); calbindin D₂₉ was a gift from Dr. S. Linse (Physical Chemistry, Lund University); S100A8, S100A9, and S100A12 were gifts from Dr. C. Kerkhof (University of Muenster); S100P was a gift from Dr. V. Gerke (University of Muenster); and S100A4 was a gift from Dr. R. Barraclough (University of Liverpool).

**Detection of S100B in Rat Brain Extract**—The rat brain was washed in phosphate-buffered saline and homogenized with a poltron homogenizer (6000 rpm) in a buffer containing 20 mM Tris-HCl, pH 7.5, 1 mM EDTA, and protease inhibitors (Complete™). The extract was then centrifuged for 15 min at 4 °C at 12,000 rpm in an Eppendorf centrifuge. The pellet was removed, and the supernatant was applied on CacyBP/SIP affinity resin (described below) after addition of CaCl₂ to a final concentration of 2 mM. Unbound proteins were washed away, and bound proteins were eluted in a buffer containing 4 mM EGTA. The EGTA-eluted fraction was analyzed on SDS gels and then on a Western blot developed with antibodies against S100A1, S100A6, or S100B. Additionally, the pellets were incubated with CaCl₂ (2 mM) or EGTA (4 mM) for 30 min at room temperature. Then, monoclonal antibodies against S100B or S100A6 were added. Incubation with antibodies and subsequently with protein G-agarose, and then washing, elution, transfer, and Western blot of immunoprecipitated proteins were performed as described above.

**CacoysBP/SIP Binds S100 Proteins**—Recombinant S100 proteins were incubated in histidine-tagged CacoysBP/SIP in a buffer containing 20 mM Tris-HCl, pH 7.5, and CaCl₂ (2 mM) or EGTA (4 mM) for 30 min at room temperature. Next, monoclonal antibodies against histidine tag were added, and incubation was continued for another 1 h at room temperature. The solutions were then incubated with protein G-agarose (Amersham Biosciences) for 1 h at room temperature. After incubation, the beads were washed four times in a buffer containing 20 mM Tris-HCl, pH 7.5, and 2 mM CaCl₂ or 4 mM EGTA. The proteins bound to protein G-agarose were eluted with a buffer containing 100 mM glycine-HCl, pH 2.7 and precipitated with cold acetone. The pellets were then solubilized in SDS sample buffer and applied on the SDS-polyacrylamide gel. After electrophoresis, the proteins were transferred to the nitrocellulose sheets and analyzed with affinity-purified antibodies against CacoysBP/SIP and monoclonal antibodies against either S100A1, S100A6, or S100B. The blots were developed with the chemiluminescent reagent (Pierce).
proteins were electrophoretically transferred from the polyacrylamide gel to the nitrocellulose sheets. The blots were washed three times for 10 min in 50 mM Tris-HCl, pH 7.5, and blocked for 2 h in 50 mM Tris-HCl, pH 7.5, 4% bovine serum albumin, 1 mM CaCl$_2$ (or 2 mM EGTA). The nitrocellulose sheets were then incubated for 1 h in 50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 1% bovine serum albumin, 1 mM CaCl$_2$ (or 2 mM EGTA) supplemented with 10 $\mu$m $^{125}$I-labeled CacyBP/SIP (specific radioactivity 0.45 mCi/mmol). Subsequently, the blots were washed three times for 20 min in the incubation buffer, but without CacyBP/SIP, dried, and exposed to Amersham Hyperfilm$^TM$ MP for 72 h.

Affinity Chromatography—Affinity resin was made by coupling CacyBP/SIP or CacyBP/SIP-(155–229) to CNBr-Sepharose. Then it was placed into microcentrifuge filter tubes (0.45 $\mu$m, Sigma) and equilibrated with a buffer containing 20 mM Tris-HCl, pH 7.5, 1 mM CaCl$_2$, and 1 mM phenylmethylsulfonyl fluoride. Purified Ca$^{2+}$-binding proteins (5 $\mu$g) were dissolved in the equilibration buffer and incubated with affinity resin for 15 min at room temperature. The unbound fraction was removed by centrifugation, and the resin was washed five times with the aforementioned buffer. Bound proteins were then eluted from the affinity resin with a buffer containing 2 mM EGTA. The unbound fraction, the final (fifth) wash, and the fraction eluted with EGTA were analyzed by SDS-PAGE.

Fluorescence Spectroscopy—CacyBP/SIP was labeled with a fluorescent probe, acrylodan (Molecular Probes), using the following procedure. 400 nmol of acrylodan were added to 20 nmol of CacyBP/SIP in 10 mM Tris-HCl, pH 7.5. The reaction was performed for 2 h at room temperature, and the excess of acrylodan was removed on a PD10 column (Amersham Biosciences). The reaction mixture and the labeled protein were protected from light. Fluorescence emission spectra of CacyBP/SIP labeled with acrylodan were recorded on a Fluorolog 3 instrument (Jobin Yvon/SPEX). 1 $\mu$mol solution of acrylodan-labeled CacyBP/SIP in 10 mM Tris-HCl, pH 7.5, in the presence of 1 mM CaCl$_2$ or 2 mM EGTA was excited at 395 nm, and the emission spectra were collected from 420 to 570 nm. Buffer emission was then subtracted. Next, S100 proteins were added in a 3-fold molar excess, and the spectra were recorded again. Fluorescence of S100 proteins was subtracted, and the spectra were corrected for dilution.

Other Methods—Electrophoresis on 15% polyacrylamide gels containing 0.1% SDS was performed using the method of Laemmli. Gels were stained with Coomassie Brilliant Blue, or the proteins were transferred from the gel to a nitrocellulose sheet and detected using specific antibodies. Recombinant CacyBP/SIP or CacyBP/SIP-(155–229) were coupled with CNBr-activated Sepharose 4B (Amersham Biosciences) using the procedure outlined by the manufacturer.

RESULTS

Identification of S100B as a CacyBP/SIP Ligand in the Brain—To search for CacyBP/SIP ligand in the brain we performed affinity chromatography with CacyBP/SIP coupled to CNBr-Sepharose. Supernatant after centrifugation of the brain protein extract was applied onto CacyBP/SIP affinity resin in the presence of CaCl$_2$. The EGTA-eluted fraction contained a major protein band with a molecular mass of 10 kDa as seen on SDS-PAGE (Fig. 1). Three samples from this fraction containing the same amount of protein were separated by SDS-PAGE, transferred onto the nitrocellulose sheets, and developed with antibodies against S100A1, S100A6, or S100B. As shown in Fig. 1, antibodies against S100B recognized the 10 kDa protein, indicating that S100B from brain extract binds to CacyBP/SIP.

Interaction of CacyBP/SIP with Members of the S100 Protein Family—We next checked what members of the S100 family were able to bind to CacyBP/SIP in vitro using the nitrocellulose overlay technique combined $^{125}$I-labeled CacyBP/SIP protein. Fig. 2 (upper part) shows that the same amount of each Ca$^{2+}$-binding protein analyzed was applied on the polyacrylamide gel, blotted onto the nitrocellulose sheets, and subsequently used in the overlay experiment. As shown in Fig. 2, acrylodan-labeled CacyBP/SIP. Acrylodan-labeled CacyBP/SIP sample was excited at 395 nm, and the emission spectrum was recorded (solid lines). The S100 proteins indicated were next added in 3-fold molar excess in the presence of 1 mM of CaCl$_2$ (dashed lines) or 2 mM of EGTA (dotted lines), and the spectra were recorded again.
CacyBP/SIP Binds S100 Proteins

S100A6 and CacyBP/SIP were both identified in EAT cells where they are present at a high level (16, 19). However, in other cells or tissues the amount of each protein is significantly different. For instance, we showed that in the brain S100A6 is expressed at a very low level (25, 26), but CacyBP/SIP is expressed at a very high level (20, 23). Therefore, in the present work we looked for other S100-like protein ligands of CacyBP/SIP in the brain. We prepared CacyBP/SIP affinity resin and performed a chromatography assay with brain protein extract. We found that protein(s) bound to CacyBP/SIP affinity resin have a molecular mass of approximately 10 kDa and that this 10-kDa protein band seen on an SDS gel represents the S100B protein. Using a nitrocellulose overlay assay with \( ^{125}\text{I}-\text{CacyBP/SIP} \) and CacyBP/SIP affinity chromatography we found that S100A1, S100A6, S100A12, and S100P bind to CacyBP/SIP in a \( \text{Ca}^{2+} \)-dependent manner. This binding seems to be specific, because some S100 proteins such as S100A4 and calbindin D9k did not bind at all. Other EF-hand \( \text{Ca}^{2+} \)-binding proteins such as calmodulin and parvalbumin did not exhibit any affinity for CacyBP/SIP. The binding of purified CacyBP/SIP with S100A1 or S100B was confirmed by fluorescence spectroscopy and by co-immunoprecipitation of purified proteins. We also showed that CacyBP/SIP was co-immunoprecipitated with S100B from

**DISCUSSION**

To examine whether S100 proteins interact with CacyBP/SIP in the cell or tissue extracts, co-immunoprecipitations using S100B or S100A6 antibodies were performed. We found that in the presence of \( \text{CaCl}_2 \), CacyBP/SIP co-immunoprecipitated with S100B from brain extract and with S100A6 from EAT cell extract (Fig. 5B). Under both \( \text{CaCl}_2 \) and EGTA conditions, antibodies against S100B immunoprecipitated antigen present in brain extract. However, because antibodies against S100A6 recognize only the \( \text{Ca}^{2+} \)-bound form of S100A6, they did not immunoprecipitate S100A6 from the extract prepared in the presence of EGTA (Fig. 5B). These data show that in situ, CacyBP/SIP might form a complex with S100 proteins present in a given cell type.

**Fig. 5. Western blot of proteins after co-immunoprecipitation assays.** A, co-immunoprecipitation of purified S100A1, S100A6, or S100B with histidine-tagged CacyBP/SIP using antibodies against histidine tag. Equal amounts of each protein (5 \( \mu \)g) were used for each individual co-immunoprecipitation experiment. The Western blot was developed with affinity-purified antibodies against CacyBP/SIP (1:100 dilution) and with monoclonal antibodies against S100A1 (1:500), S100A6 (1:500), or S100B (1:500) to detect S100 protein in the immunoprecipitates. B, co-immunoprecipitation of CacyBP/SIP with S100B or S100A6 from brain and EAT cell extracts, respectively (1500 \( \mu \)g of total protein was used for each co-immunoprecipitation). Proteins from brain or EAT cell extracts were immunoprecipitated in the presence of 2 mM \( \text{CaCl}_2 \) or 4 mM EGTA using specific antibodies against S100B or S100A6. The immunoprecipitated proteins were separated by SDS-PAGE, blotted, and identified with monoclonal antibodies against S100B (1:500) or S100A6 (1:500) and affinity-purified polyclonal antibodies against CacyBP/SIP (1:100).

**Fig. 6.** Schematic representation of CacyBP/SIP indicating the localization of the Siah-1, Skp1, and S100 binding domains (aa, amino acids).

| Siah1-binding (aa 1-80) | putative Skp1-binding (aa 73-155) | S100-binding (aa 155-229) |
|------------------------|-------------------------------|------------------------|
| N                      |                               | C                      |
the brain extract in the presence of Ca\(^{2+}\). A very low expression of S100A1 and S100A6 in the brain is probably the reason why only S100B was co-immunoprecipitated with CacyBP/SIP from the brain extract and why only S100B was detected in the fraction eluted in the EGTA-containing buffer from CacyBP/SIP affinity resin (Fig. 1). Nevertheless, we hypothesize that in the brain interaction between CacyBP/SIP and S100B, S100A1, or S100A6 are of physiological significance. Since CacyBP/SIP is present in various tissues and S100 proteins exhibit cell- and tissue-specific expression, CacyBP/SIP might form a physiologically relevant complex with different S100 proteins present in abundance in different cell types.

In the present work we established that the interaction between CacyBP/SIP and S100 proteins occurs via the C-terminal fragment of CacyBP/SIP (residues 155–229), which was previously shown to contain the S100A6 binding domain (21). S100 binding domains in other targets of S100 proteins have also been identified, for instance in annexin I and II that bind about 15 residues at the N terminus of the annexins and form an amphiphilic \(\alpha\)-helix similar to those found in calmodulin-binding proteins (29). Our theoretical PSIpred analysis, performed according to Altschul et al. (30), suggests that the C-terminal fragment of CacyBP/SIP (residues 189–201) can also form an amphiphilic \(\alpha\)-helix. While the amphiphilic \(\alpha\)-helices in annexins are specific, since each helix binds only one type of S100 protein (31), the C-terminal fragment of CacyBP/SIP binds several S100 proteins (present work). To reveal the mechanism underlying the difference in specificity of CacyBP/SIP and annexins toward S100 proteins, structural studies of CacyBP/SIP-S100 complexes should be performed and the data compared with that obtained for annexin-S100 protein complexes (31, 32).

Matsuzawa and Reed (1) reported that SIP, a component of a novel ubiquitinylatation pathway leading to \(\beta\)-catenin degradation, binds Siah-1 via an N-terminal region (residues 1–80) and Skp1 via a C-terminal region (residues 78–228) (Fig. 6). Our results presented in this work show that CacyBP/SIP (155–229) binds S100 proteins. Since both Skp1 and S100 binding sites are located in the C-terminal part of CacyBP/SIP, we hypothesize that Skp1 binds to the same region to which S100 proteins bind and therefore that these two proteins compete for CacyBP/SIP. However, we have obtained preliminary results showing that the Skp1 binding region does not overlap with the S100 binding region. Thus, we suggest that the Skp1 binding region is located between the Siah-1 binding domain (residues 1–80) and the C-terminal domain of CacyBP/SIP (residues 155–229) that binds S100 proteins, presumably spanning residues 78–155 (Fig. 6).

What the physiological effect of S100 proteins on the Siah-1-CacyBP/SIP-Skp1 ubiquitinylatation complex might be remains to be established. It has been shown that this complex, besides SCF, is involved in \(\beta\)-catenin degradation (1). The degradation of \(\beta\)-catenin in some tumors is deficient (33), and the expression of S100 proteins in tumor cells is up-regulated and in some cases correlates with metastatic activity (34, 35). If S100 proteins affect the Siah-1-CacyBP/SIP-Skp1 ubiquitinylatation complex regulating \(\beta\)-catenin degradation, one may speculate that Ca\(^{2+}\)-binding proteins of the S100 family link Ca\(^{2+}\)-homeostasis with ubiquitinylatation and degradation processes. Further studies concerning the degradation of \(\beta\)-catenin via the ubiquitinylatation complex involving Siah-1-CacyBP/SIP-Skp1 and S100 proteins might help in understanding the mechanism of tumorigenesis.

Acknowledgment—We thank Dr. W. Lesniani for critical reading of our manuscript.

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