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Tubulin binding protein, CacyBP/SIP, induces actin polymerization and may link actin and tubulin cytoskeletons

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ABSTRACT

CacyBP/SIP, originally identified as a S100A6 target, was shown to interact with some other S100 proteins as well as with Siah-1, Skp1, tubulin and ERK1/2 kinases (reviewed in Schneider and Filipek, Amino Acids, 2010). Here, we show that CacyBP/SIP interacts and co-localizes with actin in NB2a cells. Using a zero-length cross-linker we found that both proteins bound directly to each other. Co-sedimentation assays revealed that CacyBP/SIP induced G-actin polymerization and formation of unique circular actin filament bundles. The N-terminal fragment of CacyBP/SIP (residues 1–179) had similar effect on actin polymerization as the entire CacyBP/SIP protein, while the C-terminal one (residues 178–229) had not. To check the influence of CacyBP/SIP on cell morphology as well as on cell adhesion and migration, a stable NIH 3T3 cell line with an increased level of CacyBP/SIP was generated. We found that the adhesion and migration rates of the modified cells were changed in comparison with the control ones. Interestingly, the co-sedimentation and proximity ligation assays indicated that CacyBP/SIP could simultaneously interact with tubulin and actin, suggesting that CacyBP/SIP might link actin and tubulin cytoskeletons.

1. Introduction

CacyBP/SIP was discovered as a ligand of a calcium binding protein, S100A6 (calcyclin) [1,2]. Later, it was found that CacyBP/SIP was expressed in various rodent tissues and cells, especially in brain and spleen [3]. Up to now, a lot of work was performed to establish the biochemical properties of CacyBP/SIP, such as the nature of its interaction with target proteins or its cellular and subcellular localization [4]. Regarding CacyBP/SIP targets, it has been shown that this protein interacts, through its C-terminal fragment, not only with S100A6 but also with some other calcium binding proteins of the S100 family [5] but so far the physiological relevance of these interactions has not been established. Parallel studies revealed that CacyBP/SIP also binds Siah-1 and Skp1, components of ubiquitin ligase [6], and that through this binding CacyBP/SIP might be involved in β-catenin degradation. The number of proteins interacting with CacyBP/SIP is still growing and now it is known that CacyBP/SIP interacts also with tubulin [7] and ERK1/2 kinases [8]. Interaction of CacyBP/SIP with tubulin suggests its involvement in reorganization of microtubular cytoskeleton. Indeed, it has been shown that CacyBP/SIP promotes differentiation of rat neonatal cardiomyocytes [9] and that the level of CacyBP/SIP increases during differentiation of neuroblastoma NB2a cells [7].

Differentiation of neuronal cells includes several phenomena such as neurite outgrowth or synaptogenesis [10] which depend not only on microtubule dynamics but also on the reorganization of actin cytoskeleton, which is regulated by a panoply of actin-binding proteins. In cells actin exists in two major pools, the monomeric and filamentous ones, and various specialized actin-binding proteins are responsible for the precise spatio-temporal regulation of the state of actin polymerization/depolymerization and/or in consequence in filament organization. Among the proteins controlling the pool of polymerized actin are profilin, the neuronal Wiskott-Aldrich syndrome protein (N-WASP)/Scar, the Arp2/3 complex, ADF/cofilin and gelsolin [11]. *In vivo* actin filaments (microfilaments) can be organized into a number of discreet structures such as filopodia, lamellipodia or stress fibers [12,13].

During the differentiation process, especially in growth cone pathfinding, the structural/physical interaction between the actin cytoskeleton and microtubules seems to play an important role. This interaction can be mediated by different classes of proteins, including molecular motors and their complexes [14,15], conventional microtubule-associated proteins [16,17] and spectraplakins [18]. In general,

Abbreviations: BSA, bovine serum albumin; CacyBP/SIP, calcyclin (S100A6) binding protein and Siah-1 interacting protein; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IPTG, isopropyl-1-thio-β-D-galactopyranoside; MAPs, microtubule-associated proteins; MEM, minimal essential medium; NB2a, mouse neuroblastoma cell line; NIH 3T3, mouse fibroblast cell line; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; PLA, proximity ligation assay; SDS, sodium dodecyl sulfate; SNHS, *N*-hydroxysulfosuccinimide

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these proteins cross-link microtubules and actin filaments by binding to microtubules along their entire length. Some other proteins such as CLIP-170 link actin with microtubules by binding to the plus ends of microtubules (+TIPs) [19,20].

In 2007, we showed that CacyBP/SIP interacted with tubulin [7]. In the present work we show for the first time that CacyBP/SIP binds also to actin, induces its polymerization and microfilament bundling, and co-localizes with actin in neuroblastoma NB2a cells. We also show that CacyBP/SIP has an effect on cell adhesion and migration. Moreover, we show that CacyBP/SIP interacts simultaneously with actin and tubulin, which suggests that CacyBP/SIP might serve as a linker protein between microtubules and microfilaments.

2. Materials and methods

2.1. Culture of neuroblastoma NB2a cells and NIH 3T3 fibroblasts

Mouse neuroblastoma NB2a cells and NIH 3T3 fibroblasts were purchased from the American Type Culture Collection. NB2a cells were grown in MEM containing 2 mM L-glutamine, 1 mM sodium pyruvate, 25 mM sodium bicarbonate, 10% fetal bovine serum, 0.1 mM non-essential amino acids, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μg/ml gentamicin and 0.25 μg/ml fungizone. NIH 3T3 fibroblasts were grown in DMEM containing 10% bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin. Cultures were maintained in the presence of 5% CO_2 at 37 °C. The media were changed every 2–3 days and cells were passaged when confluent.

To obtain NIH 3T3 cells overexpressing CacyBP/SIP, cells were transfected as described by Schneider et al. [7] either with pcDNA3.1-CacyBP/SIP (CacyBP/SIP(+)) or pcDNA3.1 alone (control). Stably transfected NIH 3T3 fibroblasts were selected based on their G418 resistance (1.6 mg/ml for 1 week and 1 mg/ml thereafter). The CacyBP/SIP expression level was tested by Western blot developed as described below using mouse monoclonal anti-CacyBP/SIP antibody.

To induce differentiation, NB2a cells were grown for 24 h in a medium with 5% fetal bovine serum, and either supplemented with bovine serum albumin (BSA) and palmitoylcarnitine at a final concentration of 50 μM and 100 μM, respectively (differentiated cells) or with BSA alone (undifferentiated cells used as a control). A stock solution of 1 mM palmitoylcarnitine in 0.5 mM BSA in PBS was prepared according to Nalecz et al. [21].

2.2. Protein purification

For expression of recombinant CacyBP/SIP or its fragments the following plasmids were used: pET28a-CacyBP/SIP [22], pET28a-CacyBP/SIP(1–179) and pET28a-CacyBP/SIP(178–229) [7]. At first, bacteria were cultured until OD_{600} reached ~0.8 and then IPTG was added to a final concentration of 0.4 mM. After 4 h bacteria were harvested and lysed using a French press in a buffer containing 50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl, 10 mM imidazole and protease inhibitors (Protease inhibitor cocktail, Sigma). All steps of protein purification were performed as described by Filipek et al. [5]. For further studies, CacyBP/SIP or its fragments were dialyzed against an appropriate buffer, centrifuged for 75 min at 100,000 ×*g* at 4 °C and only the proteins from the supernatant fraction were used for the experiments.

Actin was prepared from rabbit skeletal muscles and purified according to Spudich and Watt [23]. It was stored in the form of G-actin in a buffer containing 2 mm HEPES, pH 7.6, 0.2 mM ATP, 0.1 mM $CaCl_2$, 0.2 mM DTT and 0.02% NaN_3 (buffer G). For cross-linking experiments, actin was prepared and stored in the G buffer without DTT.

Tubulin was purified from the cytosolic fraction of fresh porcine brain tissue according to the method of Mandelkow et al. [24] with some modifications described by Nieznanski et al. [25]. Purified tubulin was stored at −70 °C and before experiments was thawed and centrifuged as described below in the Sedimentation experiments section.

2.3. Cross-linking experiments

CacyBP/SIP was mixed with G-actin at final concentrations of 10 μM and 20 μM, respectively, in 80 μl of a buffer containing 2 mM HEPES, pH 7.6, 0.5 mM ATP and 0.2 mM $CaCl_2$. EDC and SNHS were added to a final concentration of 5 mM and 2.5 mM, respectively. After 1 h incubation at 25 °C, the reaction was terminated by addition of DTT to a final concentration of 11 mM. One fifth of the reaction mixture was analyzed by SDS-PAGE with a 10% separating SDS gel according to Laemmli [26].

2.4. Affinity chromatography

The affinity resin was prepared by coupling CacyBP/SIP to CNBr-Sepharose (Fluka) following the procedure outlined by the manufacturer. The extract from NB2a cells was prepared as follows: NB2a cells were washed twice with phosphate-buffered saline (PBS), harvested and homogenized mechanically 30 times in the affinity chromatography buffer (AC) containing 10 mM Tris, pH 7.5, 50 mM KCl, 3 mM $MgCl_2$, 0.1% Triton X-100 and protease inhibitors (Protease inhibitor cocktail, Complete Mini EDTA-free, Roche) using a syringe with a needle (25-gauge; 0.5 × 15). The extracts were centrifuged for 10 min at 12,000 rpm at 4 °C in an Eppendorf centrifuge. Supernatant was then applied to the Sepharose resin coupled with BSA to eliminate the non-specific binding. The unbound fraction eluted in the AC buffer was then applied to the Sepharose-CacyBP/SIP resin. The resin was washed with the AC buffer and then with the AC buffer containing 150 mM KCl. After an extensive washing bound proteins were eluted with the AC buffer containing 500 mM KCl, concentrated on Centricon-30 (Amicon) and analyzed by SDS-PAGE and Western blotting using anti-actin antibody. Then the same blot was analyzed using anti-GAPDH antibody in order to check the binding specificity.

2.5. Sedimentation experiments

The effect of CacyBP/SIP (or its fragments) on actin polymerization was assessed in two types of sedimentation experiments. In the first one we checked whether CacyBP/SIP induced actin filament formation and for that CacyBP/SIP was added to G-actin (10 μM) in two molar ratios (1:2 and 1:4; final concentration of CacyBP/SIP—2.5 and 5 μM) in the G buffer. In the second experiment, we checked whether CacyBP/SIP interacted with actin in actin polymerizing conditions and in this case CacyBP/SIP and G-actin were incubated in the G buffer supplemented with 2 mM $MgCl_2$ and either 50, 100 or 500 mM KCl. The protein mixture was incubated for 30 min at RT and then the sample was centrifuged for 1 h at 235,000 ×*g* at 4 °C. The pellets were resuspended in an equal volume of a buffer used for the reaction and (together with the supernatant) analyzed on SDS-PAGE. In all cases, prior to the experiment, CacyBP/SIP and its fragments were dialyzed against a buffer containing 5 mM HEPES, pH 8.0. Then, all proteins used in the experiments were cleared by 1 h centrifugation at 235,000 ×*g* at 4 °C.

To check whether CacyBP/SIP may simultaneously bind tubulin and actin, a co-sedimentation assay with these three proteins was performed. CacyBP/SIP and tubulin were pre-incubated for 10 min at RT and then added to pre-formed F-actin and incubated for another 45 min at RT in the G buffer supplemented with 100 mM KCl and 2 mM $MgCl_2$. Then, samples were centrifuged for 45 min at 235,000 ×*g* at 4 °C. Control samples were prepared in the same way but one (or two) proteins were omitted. Final concentrations of CacyBP/SIP, tubulin and actin used in the experiment were 10 μM, 2.5 μM and 10 μM, respectively. The pellets were resuspended in an equal volume of the buffer used for the reaction and both the

supernatant and pellet fractions were analyzed on SDS-PAGE. Prior to the experiment, CacyBP/SIP was dialyzed against a buffer containing 5 mM HEPES pH 8.0 and 100 mM KCl and then all proteins used in the reaction were cleared by 45 min centrifugation at 235,000 ×*g* at 4 °C.

Dissociation constant K_d was determined in the co-sedimentation experiments performed substantially as described above in the G buffer supplemented with 2 mM $MgCl_2$ and KCl at a final concentration of 50 or 100 mM. In these experiments, pre-formed F-actin (4 μM) was incubated with increasing concentrations of CacyBP/SIP (from 0.5 to 4 μM final concentration). For control experiments, sedimentation of CacyBP/SIP without actin was performed and results were included into the calculation. The obtained data were fitted to the binding equation:

$$\text{CacyBP/SIP}_{\text{bound}} = [\text{F} - \text{actin}] * [\text{CacyBP/SIP}_{\text{free}}] / (K_d + [\text{CacyBP/SIP}_{\text{free}}])$$

where [F-actin] represents the concentration of filamentous actin. The presumption was that the binding between actin and CacyBP/SIP is 1:1 (mole:mole).

2.6. Transmission electron microscopy

Actin, at 2 μM final concentration, was incubated in the G buffer or in the G buffer supplemented with 2 mM $MgCl_2$ and 50 or 100 mM KCl in the absence or presence of 1 μM CacyBP/SIP. The incubation was carried out for 30 min at 37 °C. Copper grids (400 mesh) covered with parlodion (SPI Supplies) and carbon were used. Ten microliters of samples was applied to a grid for 40 s. Negative staining was performed with 2% (w/v) aqueous solution of uranyl acetate (SPI Supplies) for 25 s. The grids were examined in a JEOL-1200EX electron microscope at an accelerating voltage of 80 kV with a 50 μm objective aperture.

2.7. Immunofluorescence microscopy

NB2a cells were grown on coverslips pretreated with 50 μg/ml of poly-L-lysine (Sigma) and then fixed with 3% paraformaldehyde in PIPES buffer (120 mM PIPES, 50 mM HEPES, 20 mM EGTA, 2 mM $MgCl_2$) for 20 min at RT. The coverslips were then washed with PBS, incubated with 50 mM NH_4Cl in PIPES buffer for 10 min at RT, washed with PBS and then cells were permeabilized for 4 min with 0.1% Triton X-100 in PIPES buffer. After washing with PBS, cells were incubated for 1 h with 3% BSA in PBS and subsequently incubated for 1 h with anti-CacyBP/SIP serum (1:500) [22]. Cells were then washed three times for 10 min in PBS and incubated for 1 h with Alexa Fluor 488 goat anti-rabbit IgG (H + L) antibodies (1:200) (Molecular Probes). After that cells were washed three times with PBS and incubated for 1 h with 1% BSA in PBS and for 1 h with TRITC-phalloidin (1:5000) (Sigma). Cells were washed three times for 10 min in PBS and mounted on glass slides with Vectashield mounting medium (Vector Laboratories). Cells were analyzed in a Leica microscope (TCS SP5 Spectral Confocal with STED).

For co-localization studies of CacyBP/SIP, tubulin and actin, cells were cultured and fixed as described in Sections 2.1 and 2.7. To visualize the areas in which CacyBP/SIP interacts with tubulin, the proximity ligation assay (PLA, Olink) was employed. The reaction with primary antibodies was carried out at RT for 1.5 h using rabbit monoclonal anti-tubulin (1:250) (Epitomics) and mouse monoclonal anti-CacyBP/SIP (1:120) (Abcam) antibodies. All other steps were performed according to manufacturer's instructions. To visualize actin cytoskeleton, cells were incubated for 1 h at RT with Alexa Fluor 488–phalloidin (1:200) (Invitrogen) in 1% of BSA in PBS. Then cells were washed with PBS followed by a series of SSC buffers and by 70% ethanol, and mounted on glass slides. All steps were performed according to manufacturer's instruction and with the reagents and media included in the PLA-kit. Co-localization of signals from the PLA reaction with Alexa Fluor 488–phalloidin was calculated using the LAS AF (Leica) program with a co-localization module employing threshold and background parameters of 80% and 20%, respectively for both stainings.

Stably transfected NIH 3T3 fibroblasts were cultured and fixed as described for NB2a cells and after washing with PBS cells were incubated for 1 h with 3% BSA in PBS. Actin cytoskeleton was visualized by incubation for 1 h with TRITC-phalloidin (1:5000) (Sigma). Cells were then washed three times for 10 min in PBS and mounted on glass slides with Vectashield mounting medium (Vector Laboratories). Cells were analyzed in a Leica TCF SP2 Spectral Confocal Microscope.

2.8. Cell adhesion assay

To measure the adhesion of NIH 3T3 fibroblasts with different CacyBP/SIP level, the CytoSelect 48-Well Cell Adhesion Assay (Cell Biolabs) was applied. Cells were seeded at the density of 10^5 per well in a serum free media and the assay was performed according to the manufacturer's instructions. Sample absorbance was measured at 560 nm using a 96-well plate and a Sunrise (Tecan) multi-well spectrophotometer.

2.9. Cell motility assay

For the artificial wound healing (scratch) assay, a scratch was made with a 0.2 ml pipette tip through a monolayer of NIH 3T3 fibroblasts grown on a 35 mm dish. Cell debris was washed out and a new portion of medium was added. Images of 4 different areas along the scratch were made and the number of cells found in the central part of the scratch (corresponding to half of its original width) was counted after 24 h.

For random migration analysis, stably transfected NIH 3T3 fibroblasts were seeded on dishes coated with 15 μg/ml fibronectin (Sigma) at a density of 5×10^4 cells/well in a regular culture medium. After 24 h cells were placed in an environmental chamber (37 °C, 5% CO_2) of a Leica microscope (TCS SP5 Spectral Confocal with STED). Images were collected using a polarization contrast at 100× magnification at 10 min intervals over 5 h. To track the migration path of an individual cell, cells were manually traced for each frame using the ImageJ software. The migration paths were prepared as graphs using the Excel software (Microsoft).

2.10. SDS-PAGE and Western blotting

Gel electrophoresis with 10% (w/v) polyacrylamide containing 0.1% SDS was performed by the method of Laemmli [26]. Gels were either stained with Coomassie brilliant blue R250 or separated proteins were transferred electrophoretically onto nitrocellulose and identified using appropriate primary antibodies: mouse anti-CacyBP/SIP monoclonal antibody (1:1,000; Abcam), mouse anti-actin antibody (MP Biomedicals) and mouse anti-GAPDH antibody (Chemicon International) diluted 1:2000 and 1:10,000, respectively. After washing with TBS-T buffer (50 mM Tris, pH 7.5, 200 mM NaCl, 0.05% Tween 20) the blots were allowed to react with secondary goat anti-mouse IgG antibodies conjugated to horseradish peroxidase (1:10,000) (Jackson Immunoresearch Laboratories). After three washes with TBS-T and two washes with TBS (50 mM Tris, pH 7.5, 200 mM NaCl) blots were developed with ECL chemiluminescence kit (Amersham Biosciences) followed by exposition against an X-ray film. The intensities of the protein bands were quantified using the Ingenius densitometer and the Gene Tools Software (Syngene) with GAPDH as a reference protein.

3. Results

3.1. CacyBP/SIP co-localizes with and binds actin in NB2a cells

We have previously reported that in differentiated NB2a cells CacyBP/SIP was mainly localized in the cellular processes and that

with the exception of their distal parts, it was co-localized with tubulin [7]. Since it has been shown that several tubulin binding proteins are also able to interact with actin [27], and knowing that actin is present in the growth cones (edges) of cellular processes, we checked whether CacyBP/SIP could also interact with actin. Double immunostaining performed on NB2a cells revealed that CacyBP/SIP co-localized with actin in both undifferentiated and differentiated cells (Fig. 1A). As we expected, in differentiated NB2a cells co-localization between CacyBP/SIP and actin was observed in the distal parts of cellular processes.

To establish whether the interaction between CacyBP/SIP and actin occurs in NB2a cell extract, we performed affinity chromatography with CacyBP/SIP coupled to CNBr-Sepharose. The supernatant obtained from the protein extract of NB2a cells was first applied to Sepharose coupled with BSA and then the unbound fraction was applied to the CacyBP/SIP-Sepharose resin (Fig. 1B, lane 1). The resin was then extensively washed and the bound proteins were eluted with the same buffer supplemented with 500 mM KCl. As it can be seen in Fig. 1B (lane 3) the bound fraction contains actin but no GAPDH (a control protein).

3.2. CacyBP/SIP directly interacts with actin

To check whether CacyBP/SIP interacts directly with actin, we performed chemical cross-linking of purified proteins using EDC, a zero-length cross-linker. As it can be seen in Fig. 1C, in the presence of

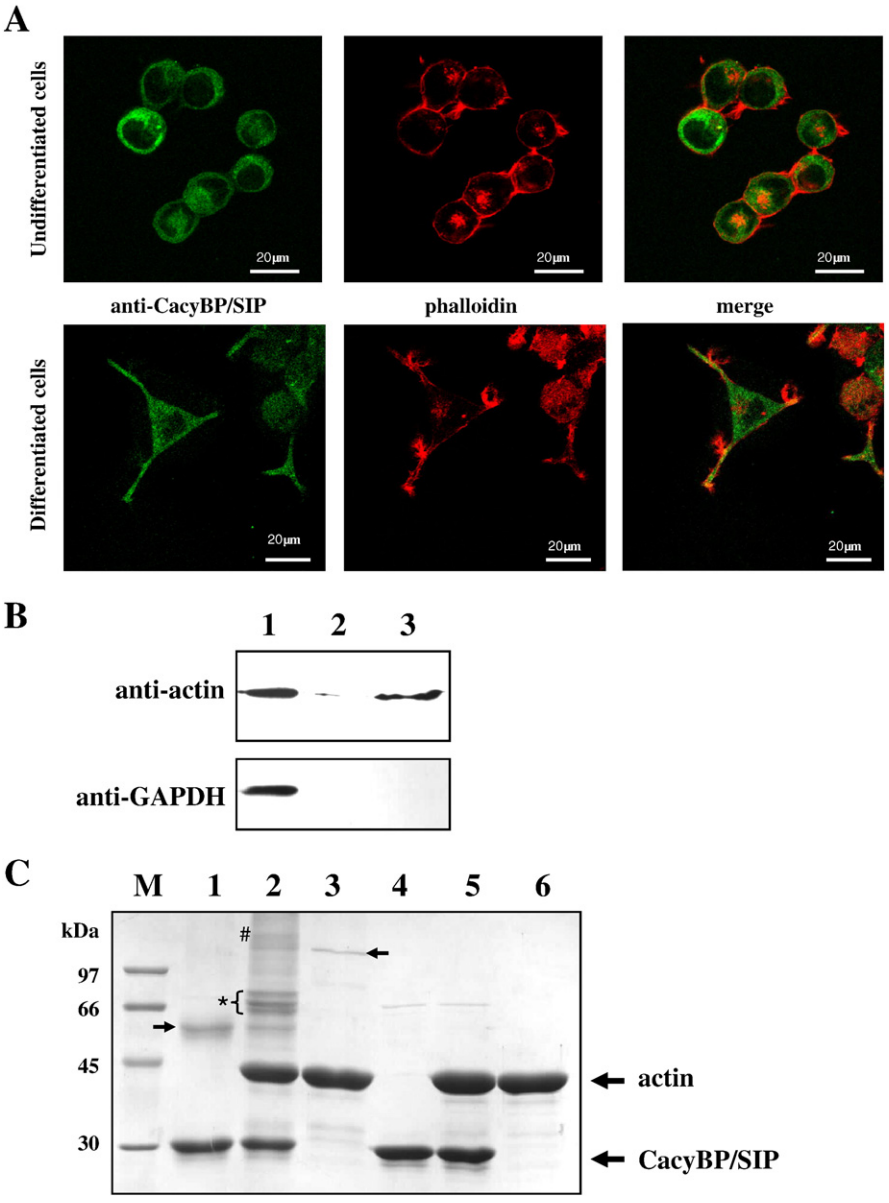

Fig. 1. Interaction of CacyBP/SIP with actin. (A) Co-localization of CacyBP/SIP and actin in NB2a cells. NB2a cells were stained with anti-CacyBP/SIP polyclonal antibodies (left panels, in green) followed by incubation with TRITC-phalloidin (middle panels, in red). Right panels (merged) present the co-localization of both proteins under plasma membrane of undifferentiated cells (upper panels) and in cellular processes of differentiated cells (lower panels). Each image represents one optical section (0.5 μm thick, pinhole 1.0) of the cells obtained with the use of confocal microscope (Leica). (B) Binding of actin to CacyBP/SIP affinity resin. Lane 1, NB2a cell extract protein (10 μg); lane 2, the last wash; lane 3, the fraction eluted from the CacyBP/SIP-Sepharose resin with buffer containing 500 mM KCl. The upper panel shows Western blot developed with anti-actin antibody and the lower panel shows the same blot developed with anti-GAPDH antibody (control). (C) Cross-linking of CacyBP/SIP with G-actin analyzed by SDS-PAGE. One fifth of the reaction mixture was applied on each lane of the SDS gel which was then stained with Coomassie brilliant blue. Lanes 1 and 4, CacyBP/SIP; lanes 2 and 5, mixture of CacyBP/SIP and actin; lanes 3 and 6, actin. Proteins were incubated with (lanes 1–3) or without (4–6) cross-linker EDC-SNHS. "*" and "#" indicate the CacyBP/SIP-actin cross-linking products; arrows indicate actin and CacyBP/SIP dimers. Lane M shows molecular weight standards.

the cross-linker CacyBP/SIP and actin formed covalent products migrating at the level of approximately 70-kDa (indicated by *) in the SDS gel. The mobility of these bands corresponds to the sum of molecular masses of CacyBP/SIP (30 kDa) and actin (42 kDa). No such bands were observed in the samples of CacyBP/SIP or actin alone incubated with the cross-linking agent. Moreover, we observed a band (indicated by #) migrating above the 97-kDa molecular weight standard, which suggests that CacyBP/SIP and actin might form additional complexes.

3.3. CacyBP/SIP induces actin polymerization and formation of actin filament bundles

Since actin and CacyBP/SIP interacted with each other, we checked whether CacyBP/SIP might induce actin polymerization. For that we performed sedimentation experiments in which CacyBP/SIP was incubated with G-actin (in the G buffer without KCl and $MgCl_2$). The samples were then centrifuged and the supernatant and pellet fractions were analyzed on the SDS gel. As it can be seen in Fig. 2A, actin alone was detected mainly in the supernatant, whereas in the presence of CacyBP/SIP, a certain amount of actin was found in the pellet, suggesting that CacyBP/SIP might induce actin polymerization. This observation was confirmed by the electron microscopy analysis. As it can be seen in Fig. 3G and H, after incubation of actin in the presence of CacyBP/SIP-actin filaments as well as bundles of actin filaments were observed, whereas in its absence no such actin structures were seen (Fig. 3A and B). As it can be seen in Fig. 2B and C, in the case of the N-terminal fragment of CacyBP/SIP, spanning residues 1–179, the pelleting of actin was observed similarly as for the entire form of CacyBP/SIP. In the case of the C-terminal fragment (residues 178–229), the pelleting of actin was not observed.

To check whether CacyBP/SIP-actin interaction is of ionic character, CacyBP/SIP and G-actin were incubated in a buffer containing different concentrations of KCl. As shown in Fig. 2D and E, CacyBP/SIP co-sediments with F-actin. Interestingly, electron microscopy analysis of actin incubated in the presence CacyBP/SIP in a buffer containing 50 mM KCl revealed that CacyBP/SIP induced formation of actin filament bundles of different shapes (Fig. 3I and J). Those bundles seemed to be very flexible as they could form loops or circular structures. In the presence of CacyBP/SIP in a buffer containing 100 mM KCl actin filaments were also present in the form of bundles (Fig. 3K and L). However, these bundles were not so flexible as those formed in the buffer containing 50 mM KCl since we observed loops but no circles. No bundles were visible when actin was incubated alone (Fig. 3C–F).

3.4. Dissociation constant of the CacyBP/SIP-actin complex

To calculate the dissociation constant (K_d), we applied a co-sedimentation method as it was described in the Section 2.5. Data obtained for three independent experiments with the assumption of CacyBP/SIP:actin binding 1:1 (mole/mole) yielded a dissociation constant of 3.22×10^{-6} for complex in the buffer containing 50 mM KCl, whereas in the buffer containing 100 mM KCl the K_d was 8.47×10^{-6} M.

3.5. Changes in adhesion and migration of NIH 3T3 fibroblasts with different CacyBP/SIP levels

Dynamic reorganization of the cytoskeleton is prerequisite for cell adhesion and migration, and since we have shown that CacyBP/SIP interacts with actin we tested whether this interaction might have physiological relevance. To check that, we generated a stable line of NIH 3T3 fibroblasts transfected with the pcDNA3.1-CacyBP/SIP expression plasmid. Fibroblasts were chosen since their cytoskeleton organization and migration rate are very well characterized. Densitometric analysis of three independent Western blots indicated that

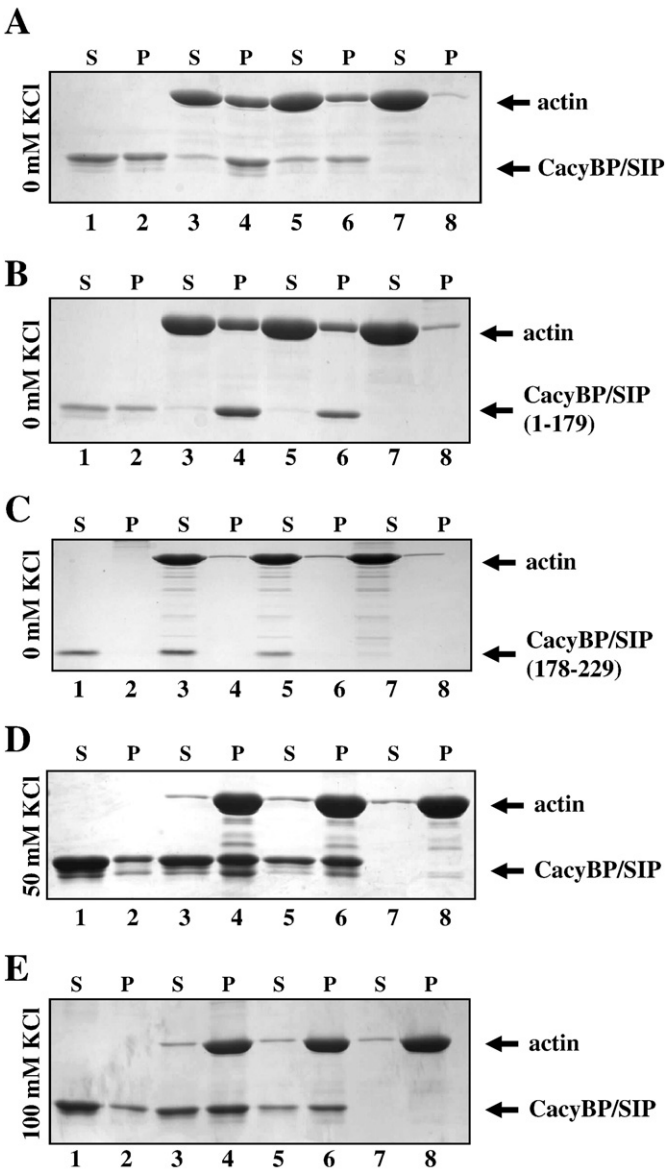

Fig. 2. CacyBP/SIP interacts with both monomeric and filamentous actin. Co-sedimentation of CacyBP/SIP or its fragments with actin was analyzed by SDS-PAGE. (A–E) CacyBP/SIP or its fragments (lanes 1 and 2), actin (lanes 7 and 8) or mixtures of CacyBP/SIP or its fragments with actin at a molar ratio 1:2 (lanes 3 and 4) or 1:4 (lanes 5 and 6) were incubated in a buffer containing 0 (A–C), 50 (D) or 100 (E) mM KCl (as indicated on the left). After centrifugation equal amounts of supernatant (S) and pellet (P) fractions were loaded on the SDS gel.

cells transfected with the plasmid exhibited 88% (±13%) higher amount of the CacyBP/SIP protein than control cells transfected with empty pcDNA3.1 plasmid (not shown). Morphological analysis of fibroblasts overexpressing CacyBP/SIP revealed that these cells did not differ significantly from control ones (data not shown).

At first, we have compared the adhesive properties of NIH 3T3 fibroblasts with normal (control) and increased CacyBP/SIP (CacyBP/SIP(+)) levels. We found that CacyBP/SIP(+) cells adhered stronger to the surfaces covered with laminin and fibrinogen than control cells (Fig. 4A). There was no difference in adhesive properties when the surface was covered with fibronectin, collagen I or collagen IV (not shown). The observed differences in adhesion of CacyBP/SIP(+) cells in comparison with control ones might be an effect of subtle cytoskeleton rearrangements in the areas of cell adhesion. Since, in general, changes in the adhesion strength might influence cell migration we have compared migratory properties of the NIH 3T3

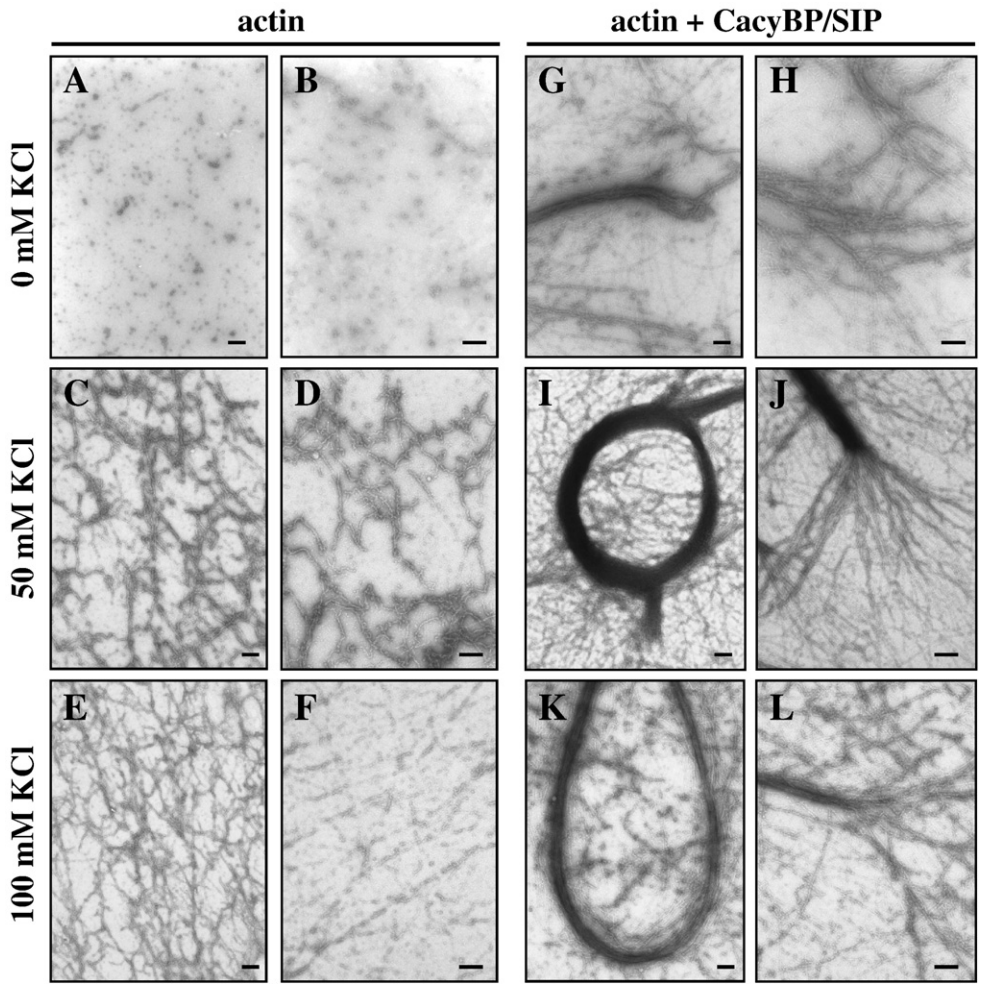

Fig. 3. CacyBP/SIP induces actin polymerization and actin filament bundling. Micrographs showing the influence of CacyBP/SIP on microfilament assembly depending on KCl concentrations. Panels A–F show actin alone and panels G–L show actin after incubation with CacyBP/SIP. Note that in a buffer without KCl actin is present in a form of globular oligomers (A and B) whereas in the presence of CacyBP/SIP numerous actin filaments can be seen (G and H). In buffers containing 50 mM (C and D) or 100 mM KCl (E and F) actin forms filaments whereas in the presence of CacyBP/SIP (I and J, and K and L, respectively) actin filament as well as actin filament bundles of different shape are visible. The scale bar is 200 nm.

fibroblasts with normal (control) or increased level of CacyBP/SIP. For that purpose we performed the artificial wound healing (scratch) assay. To keep the focus on cell motility and to minimize the influence of proliferation, we counted only the cells spotted in the central area of the scratch (corresponding to half of its original width). We found that after 24 h 1.8 times more control fibroblasts than those with increased level of CacyBP/SIP were present in the examined area (Fig. 4B). Differences in cell motility were also revealed by a random migration analysis. As it can be seen in Fig. 4C, control fibroblasts migrated for longer distances (164 ± 19.3 μm) than cells overexpressing CacyBP/SIP (116 ± 11.0 μm).

3.6. CacyBP/SIP may link actin and tubulin cytoskeletons

Our current and earlier data demonstrating that CacyBP/SIP interacts with actin and tubulin encouraged us to test whether CacyBP/SIP can simultaneously bind to both proteins. To assess this we performed sedimentation experiments using these three proteins and the proximity ligation assay (PLA) combined with Alexa Fluor 488 phalloidin staining to confirm an *in vivo* observed interaction.

Fig. 5A (panels 2, 4, 6, 7) shows that a certain amount of tubulin is seen in the pellet in the absence or in the presence of polymerized actin and/or CacyBP/SIP. Fig. 5B shows densitometric analysis performed for six independent experiments. The experiments were done in conditions, in which tubulin did not polymerize into microtubules and tubulin oligomers (formed in the presence of CacyBP/SIP) [7] did not sediment. As it can be seen in Fig. 5B, when tubulin was incubated alone or with CacyBP/SIP a similar amount of tubulin (about 7–8%) was found in the pellet fractions. The amount of tubulin present in the pellet fraction was increased up to 20% when the sample was incubated with actin. In the case of tubulin sample incubated with both actin and CacyBP/SIP, the amount of pelleted tubulin was further increased up to 25%. This suggests that a certain amount (about 5%) of tubulin in the pellet may result from binding to CacyBP/SIP which was already bound to filamentous actin, indicating a tripartite interaction. However, since actin itself increases tubulin sedimentation we cannot exclude that parallel with the tripartite complex some other complexes could be formed.

Actin-tubulin-CacyBP/SIP tripartite interaction has been confirmed by proximity ligation assay (PLA) as the immunofluorescence signals corresponding to CacyBP/SIP-tubulin and filamentous actin revealed co-localization in both undifferentiated and differentiated NB2a cells (Fig. 5C). Interestingly, the co-localization seems to be more pronounced in differentiated cells than in undifferentiated ones, thus suggesting that this interaction may be relevant *in vivo*. Altogether, our results obtained from co-sedimentation and PLA assays indicate that CacyBP/SIP might interact simultaneously with tubulin and actin.

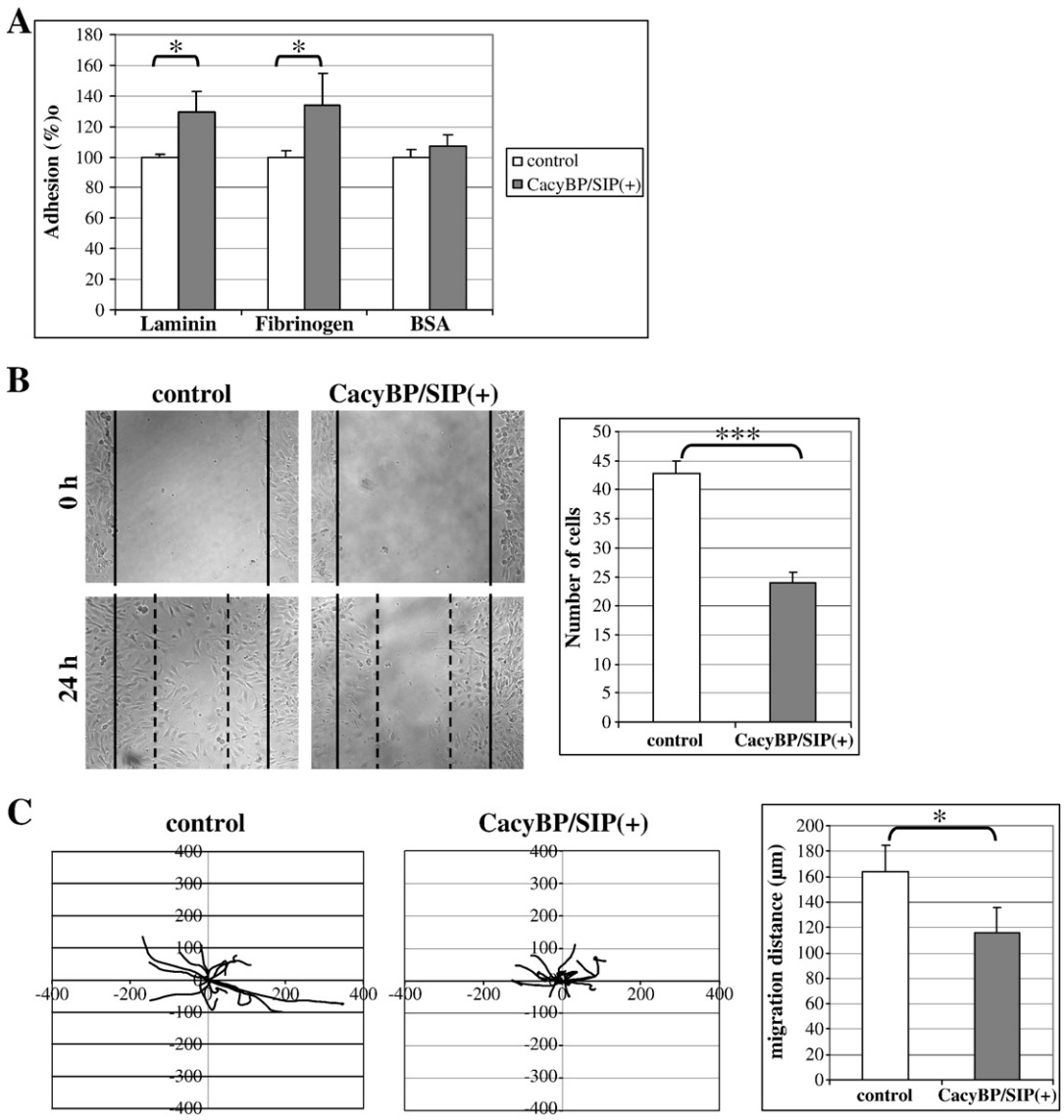

Fig. 4. Overexpression of CacyBP/SIP affects cell adhesion and migration. (A) Cell adhesion assay of control (white bars) and CacyBP/SIP(+) (grey bars) NIH 3T3 fibroblasts to laminin, fibrinogen or BSA (negative control). Cells were quantified colorimetrically at $\lambda = 560$ nm and statistical analysis was performed using Student's *t*-test. Results from four independent experiments are presented as a mean ± SEM; $^{*}P \leq 0.05$. (B). Wound healing (scratch) assay was performed on control cells and cells with increased level of CacyBP/SIP. Statistical analysis was performed using Student's *t*-test. Right panel shows the results from three independent experiments presented as a mean ± SEM; $^{***}P \leq 0.001$. (C). Random migration analysis was performed for NIH 3T3 cells with normal (control) and increased level of CacyBP/SIP (CacyBP/SIP(+)). In each case, photographs of 13 cells were taken at 100× magnification every 10 min during 5 h. Cells were manually traced on each photograph using the ImageJ software. Migration paths were prepared as graphs using the Excel software. Right panel presents the difference in migration path distances as the mean values ± SEM in µm calculated using Student's *t*-test; $^{*}P \leq 0.05$.

4. Discussion

In the present work, we have shown that CacyBP/SIP, found earlier to be a tubulin partner, binds also to actin. The co-localization of CacyBP/SIP with actin was seen in the distal parts of cellular processes of differentiated mouse neuroblastoma NB2a cells. Also, affinity chromatography revealed that actin from the protein extract of mouse neuroblastoma NB2a cells bound to the CacyBP/SIP affinity resin and was eluted in the buffer containing high salt concentration. It is noteworthy that in the eluted fraction containing actin no band corresponding to GAPDH was detected, suggesting that the CacyBP/SIP-actin binding was specific. A direct interaction between both proteins was also shown in an experiment with a zero-length cross-linker, EDC. The binding constant estimated for the CacyBP/SIP-actin complex was 3.22×10^{-6} M and 8.47×10^{-6} M in buffers containing 50 and 100 mM KCl, respectively. These values indicate that the interaction between both proteins is relatively strong and comparable to the interaction of several known actin-binding proteins such as tropomyosin [28] and avian gizzard and nonmuscle caldesmon [29]. It also seems that the CacyBP/SIP-actin interaction has an electrostatic character, similarly to other actin-binding proteins, including myosins [11]. This has been further confirmed by the co-sedimentation assay in the buffer containing 500 mM KCl, [see Fig. 1S], demonstrating that interaction of CacyBP/SIP with actin is barely noticeable.

The sedimentation assays performed in conditions favoring monomeric actin showed that CacyBP/SIP induced actin polymerization, which was further confirmed by a transmission electron microscopy. When the assays were done in conditions inducing actin polymerization (i.e. the presence of 50 or 100 mM KCl and 2 mM Mg^{2+}), co-sedimentation of CacyBP/SIP with actin filaments was still observed. Interestingly, the electron microscopy revealed formation of various forms of actin bundles, including unique circular structures. Bundle formation, which was manifested in our experimental conditions by a sample opalescence, excluded the employment of the traditional methods widely used for

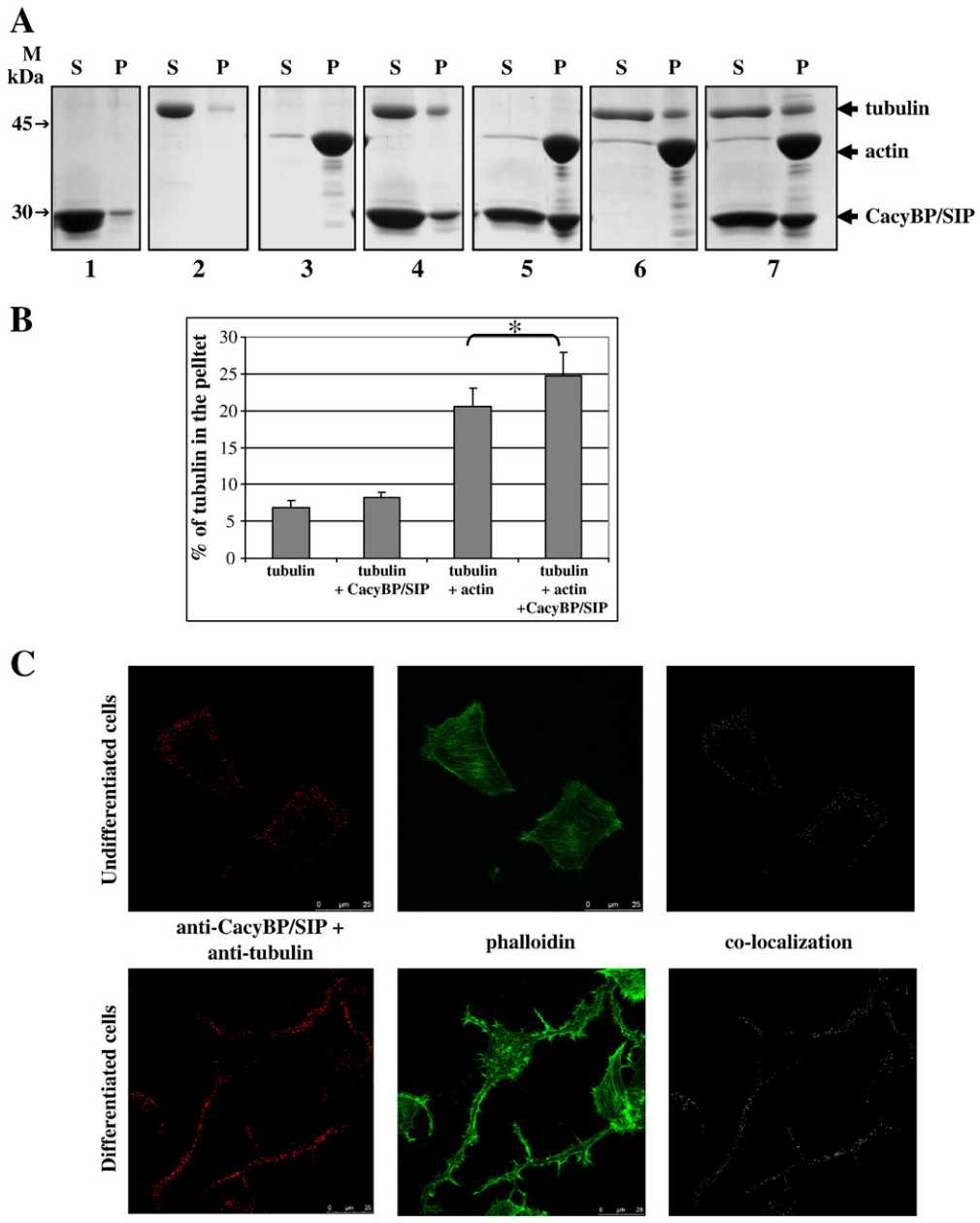

Fig. 5. Interaction of CacyBP/SIP with both tubulin and actin. (A) Coomassie staining of the SDS gel showing the sedimentation of tubulin in the presence of actin alone or of actin and CacyBP/SIP. Samples containing CacyBP/SIP (panel 1), tubulin (panel 2), F-actin (panel 3), CacyBP/SIP and tubulin (panel 4), CacyBP/SIP and F-actin (panel 5), tubulin and F-actin (panel 6) or CacyBP/SIP together with tubulin and F-actin (panel 7) were incubated and centrifuged as described in the Materials and methods section. Equal amounts of supernatant (S) and pellet (P) fractions were loaded on the gel. (B) Statistical analysis of tubulin present in the pellet calculated using Student's *t*-test from six co-sedimentation experiments. Results are presented as a mean ± SEM; $^{*}P \leq 0.05$. (C) Immunofluorescence studies using proximity ligation assay (PLA, Duolink) showing co-localization of CacyBP/SIP, tubulin and actin in NB2a cells. Co-localization of CacyBP/SIP and tubulin assessed with the PLA technique is seen in the left panels, actin cytoskeleton stained with Alexa Fluor 488 phalloidin is shown in the middle panels and co-localization of the three proteins is shown in the right panels. The co-localization is seen under the plasma membrane of undifferentiated cells (upper panels) and in cellular processes of differentiated cells (lower panels). Data were obtained using the co-localization module in LAS AF (Leica) program. Each image represents one optical section (0.3 μm thick, pinhole 1.0) of the cells obtained with the use of confocal microscopy.

monitoring actin polymerization, such as light scattering and measuring changes in the fluorescence of pyrenyl-labeled actin. Therefore we do not know whether CacyBP/SIP could affect the nucleation and elongation steps of the polymerization process.

Several actin-binding proteins known as actin cross-linkers are involved in the formation of cellular actin structures such as the microfilament network present in lamellipodia (for example filamin, spectrin or transgelin) or tightly bound actin filament bundles seen in filopodia (for example vilin, fimbrin, α-actinin, formins) [13]. The organization of actin filaments in bundles formed in the presence of CacyBP/SIP seems to be similar to that induced by fimbrin [30,31] or vilin [32], in contrast to the loosely packed filament network observed in the presence of filamins [33,34]. Interestingly, in the presence of CacyBP/SIP the bundles of actin filaments formed unique circular structures, which were not described for any other actin-binding protein. These circular bundles were seen mainly in 50 mM KCl, whereas in 100 mM KCl the bundles seemed to be less flexible since mainly loops but not circle-shaped structures were visible. The salt-dependent effect of CacyBP/SIP on actin filament structure is not unusual. A similar effect has been shown for the ENA/VASP protein where the bundles formed at low salt concentration (15 and 50 mM) were highly ordered and tightly packed, whereas in 100 and 150 mM

KCl the bundles were loosely packed, with many single filaments emanating from the bundle termini [35]. Another actin-bundling protein, fimbrin, was also found to be involved in actin filament bundling at 30 mM concentration of KCl, while at 10 mM of KCl the formation of actin bundles was less pronounced [30]. The effect of CacyBP/SIP on actin bundle formation is also similar to that observed for myelin basic protein (MBP) [36,37].

Using the recombinant fragments of CacyBP/SIP, we were able to demonstrate that the N-terminal part of the protein was engaged in the interaction with actin (see Fig. 2). Contrary to the full-length protein and its N-terminal fragment, the C-terminal fragment, the one which binds S100A6, seems not to be involved in actin polymerization. However, it cannot be excluded that S100A6 might have an indirect effect on CacyBP/SIP-induced actin polymerization *in vivo*. It has been recently shown that decreased level of S100A6 caused changes in actin filament organization in NIH 3T3 fibroblasts [38].

Since cell migration and adhesion depend on dynamic assembly/disassembly of actin cytoskeleton, we have tested whether the observed effects of CacyBP/SIP on actin filaments *in vitro* translate into the effects *in vivo*. With the use of a stable NIH 3T3 cell line with the increased level of CacyBP/SIP, we have demonstrated that overexpression of this protein inhibited cell migration, which was assessed by the *in vitro* wound healing assay and by determining the length of the cell path. The observed restraint was accompanied by the increase in the adhesion to the surface containing laminin and fibrinogen but not fibronectin, collagen I or collagen IV, in agreement with the notion that an increase in the adhesion affects cell motility [39]. However, morphological analysis of the fibroblasts overexpressing CacyBP/SIP did not reveal any significant changes in cell morphology and microfilament organization. These observations may indicate that differences in migration and adhesion might result from subtle cytoskeleton rearrangements in the adhesive structures and their vicinity, and not from global changes within the cytoskeleton.

Results presented in this work show that both actin binds to the same N-terminal fragment of CacyBP/SIP (residues 1–179) as tubulin does [7]. This suggests that the actin and tubulin binding domains within CacyBP/SIP molecule might overlap. Nevertheless, formation of a tripartite complex is possible since CacyBP/SIP forms dimers [40] or even tetramers [6]. Indeed, results from the co-sedimentation experiments, proximity ligation assay (PLA) and CacyBP/SIP affinity chromatography, in which actin (this work) and tubulin (data presented in [7]) were eluted with buffer containing 500 mM NaCl, showed that CacyBP/SIP interacts with actin and tubulin simultaneously. All these data give an assumption to the hypothesis that CacyBP/SIP may connect microfilaments with microtubules. While up to now a plethora of actin and tubulin interacting proteins have been characterized, only very few of them were shown to directly link actin and tubulin cytoskeletons [41]. The best known example is the MAP2 protein which is able to form a tripartite complex with actin and tubulin [27]. Moreover, muscle dystrophin, known to be engaged in maintaining the integrity of sarcolemma by linking dystroglycan to actin filaments, has been recently shown to directly bind to microtubules [42]. These data support our supposition about the involvement of CacyBP/SIP in cytoskeleton organization. The question is what might be the physiological role of such tripartite complexes. It is plausible that in neuronal cells and in their cellular processes (and their termini such as nerve growth cones), where both monomeric and filamentous actin but not microtubules are visible under the plasma membrane [40], CacyBP/SIP links actin and tubulin and may play a role in the maintenance of the cytoskeleton integrity in the processes and their termini. It should be kept in mind that CacyBP/SIP is expressed predominantly in neurons and its synthesis is enhanced during differentiation [7,9], a process accompanied by cytoskeletal reorganization.

Altogether, our results showing that CacyBP/SIP interacts with actin both *in vitro* and *in vivo,* and may simultaneously bind to actin and tubulin suggest its possible involvement in regulation of cytoskeletal organization and the cytoskeleton-dependent cellular phenomena.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbamcr.2010.07.003.

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