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CacyBP/SIP interacts with tubulin in neuroblastoma NB2a cells and induces formation of globular tubulin assemblies

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Abstract

CacyBP/SIP, originally identified as a S100A6 (calcyclin) target, was later shown to interact with some other members of the S100 family as well as with Siah-1 and Skp1 proteins. Recently, it has been shown that CacyBP/SIP is up-regulated during differentiation of cardiomyocytes. In this work we show that the level of CacyBP/SIP is higher in differentiated neuroblastoma NB2a cells than in undifferentiated ones and that in cells overexpressing CacyBP/SIP the level of GAP-43, a marker of differentiation, was increased. Since the process of differentiation is accompanied by an extensive rearrangement of microtubules, we examined whether CacyBP/SIP interacted with tubulin. By applying cross-linking experiments we found that these two proteins bind directly. The dissociation constant of the tubulin–CacyBP/SIP complex determined by the surface plasmon resonance technique is 1.57×10^{-7} M which suggests that the interaction is tight. The interaction and co-localization of CacyBP/SIP and tubulin was also demonstrated by co-immunoprecipitation, affinity chromatography and immunofluorescence methods. Light scattering measurements and electron microscopy studies revealed that CacyBP/SIP, but not its homologue, Sgt1, increased tubulin oligomerization. Altogether, our results suggest that CacyBP/SIP, via its interaction with tubulin, might contribute to the differentiation of neuroblastoma NB2a cells.

Keywords: CacyBP/SIP; Tubulin; Interaction; Differentiation; Globular assemblies; NB2a cells

1. Introduction

CacyBP/SIP was discovered as a ligand of S100A6 (calcyclin) in the cytosolic fraction of Ehrlich ascities tumor cells [1]. Later, the sequence encoding this protein was cloned and specific antibodies were produced [2,3]. Using these antibodies it was found that CacyBP/SIP is expressed in various rodent tissues and cells; a particularly high level of this protein was found in brain and spleen [3]. Up to now a lot of work was performed to elucidate the properties of CacyBP/SIP such as the nature of its interaction with target proteins [4–8] or its cellular and subcellular localization [9,10]. Regarding CacyBP/SIP targets, it has been shown that this protein interacts, through its C-terminal end, with several calcium binding proteins of the S100 family [11] but, so far, there are no data describing the physiological importance of these interactions. Parallel studies revealed that CacyBP/SIP also binds Siah-1 and Skp1, components of ubiquitin ligase [5], and that through this binding CacyBP/SIP is involved in the ubiquitination and degradation of β-catenin.

Recently, Au and coworkers [12] showed that CacyBP/SIP promotes differentiation of rat neonatal cardiomyocytes. Differentiation is accompanied by extensive reorganization of the cytoskeletal structures: microtubules, actin filaments and intermediate filaments. Microtubules, linear polymers of α-/β-tubulin

Abbreviations: BSA, bovine serum albumin; CacyBP/SIP, calcyclin (S100A6) binding protein and Siah-1 interacting protein; CLIP, cytoplasmic linker protein; DTT, dithiothreitol; EB1, end-binding protein; EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; GAPDH, glyceraldehydes 3-phosphate dehydrogenase; GAP-43, growth-associated protein 43; IPTG, isopropyl-1-thio-β-D-galactopyranoside; MAPs, microtubule-associated proteins; MCAK, mitotic centromere-associated kinesin; MEM, minimal essential medium; NB2a, mouse neuroblastoma cell line; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; SNHS, *N*-hydroxysulfosuccinimide; SPR, surface plasmon resonance; TPPP/p25, tubulin polymerization promoting protein

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heterodimers are the predominant components of the cytoskeleton in neurons, cells in which CacyBP/SIP is highly expressed [9]. Stability of microtubules is regulated by interaction with many proteins referred to as microtubule-associated proteins (MAPs). Some MAPs such as Tau, MAP2, MAP1A, MAP1B, doublecortin, EB1 or CLIP-170 belong to microtubule stabilizers [13] that bridge tubulin subunits in the polymer or cap microtubule ends. Other proteins of the MAPs family, such as Op18/stathmin, katanin or MCAK, are known to destabilize microtubules [13,14]. Most MAPs bind to microtubules directly and some of them interact preferentially with tubulin heterodimers.

The finding that CacyBP/SIP is abundant in neurons and in neuroblastoma NB2a cells [3,9] and the fact that CacyBP/SIP was shown to be up-regulated during differentiation of rat cardiomyocytes [12] prompted us to check whether this protein might be involved in differentiation of NB2a cells. Since differentiation of neuronal cells is accompanied by extensive reorganization of microtubules [15], we examined whether CacyBP/SIP interacts with tubulin, a major microtubule component, and whether it affects tubulin polymerization/oligomerization.

2. Materials and methods

2.1. Culture and differentiation of neuroblastoma NB2a cells

Mouse neuroblastoma NB2a cells were grown in MEM containing 10% fetal bovine serum, 25 mM sodium bicarbonate, penicillin (100 μg/ml), streptomycin (100 μg/ml), gentamycin (50 μg/ml) and fungizon (0.25 μg/ml). 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 induce the differentiation process NB2a cells were grown for 24 h in MEM containing 5% fetal bovine serum 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 and coworkers [16].

2.2. Plasmid construction and protein purification

p3xFLAG-CMV-10-CacyBP/SIP and pcDNA3.1-CacyBP/SIP plasmids for eukaryotic cell transfection were prepared as follows. The CacyBP/SIP coding sequence was amplified by PCR using plasmid pET28a-CacyBP/SIP (prepared as described in [11]) as a template with primers: forward 5′-GCGAAGCTTATGGCTTCCGTTTTGGAAGAG-3′ and reverse 5′-CGCGGATCCTCAAAATTCCGTGTCTTCTCTG-3′. This reaction product was digested with *Hin*dIII and *Bam*HI restriction enzymes and introduced into the p3xFLAG-CMV-10 (Sigma) or pcDNA3.1 (Invitrogen) plasmids previously digested with the same enzymes. The correct sequence of the cloned inserts was confirmed by DNA sequencing.

For expression of recombinant CacyBP/SIP or its fragments the following plasmids were used: pET28a-CacyBP/SIP, pET28a-CacyBP/SIP (1–179) and pET28a-CacyBP/SIP (178–229). pET28a-CacyBP/SIP (178–229) was obtained from pET28a-CacyBP/SIP using primers: forward 5′-GAGAGCATATGCTCGAGGAAAAGCCTTCCTAC-3′ and reverse 5′-GAGACGGATCCTCAAAATTCCGTGTCTTC-3′. The reaction product was digested with *Nde*I and *Bam*HI restriction enzymes and introduced into the pET28a previously digested with the same enzymes. The correct sequence of the cloned insert was confirmed by DNA sequencing. pET28a-CacyBP/SIP (1–179) was prepared as follows. The insert from the pET30a plasmid (prepared as described in [4]) was cut using the restriction enzymes *Eco*RI and *Sal*I and introduced into the pET28a previously digested with the same enzymes.

To purify proteins, 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 French press in buffer containing 50 mM NaH_2PO_4 pH 8.0, 300 mM NaCl, 10 mM imidazole and protease inhibitors (protease inhibitor cocktail, Sigma). All other steps were performed as described in [11]. For further studies, the CacyBP/SIP protein or its fragments were dialyzed against an appropriate buffer, centrifuged for 75 min at 100,000×*g* at 4 °C and only the protein from the supernatant fraction was used for experiments.

Tubulin was purified from the cytosolic fraction of fresh porcine brain tissue according to the method of Mandelkow and coworkers [17] with some modifications described in details by Nieznanski and coworkers [18]. Purified protein was stored at −70 °C and before experiments tubulin was thawed and centrifuged for 20 min at 22,000×*g* at 4 °C. The pellet was discarded and tubulin from the supernatant fraction used in further studies.

2.3. Cross-linking experiments

CacyBP/SIP was mixed with unpolymerized tubulin at final concentrations of 21.5 μM and 40 μM, respectively, in 40 μl of the buffer containing 10 mM Tris pH 7.0, 16 mM $MgCl_2$, 1 mM GTP and 10% glycerol. EDC and SNHS were added to 5 mM and 2.5 mM final concentration, respectively. After 1-h incubation at 25 °C, the reaction was quenched 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 gel according to Laemmli [19]. The cross-linking product was excised from the gel stained with Coomassie brilliant blue and subjected to mass spectrometry analysis (Institute of Biochemistry and Biophysics, Warsaw, Poland).

2.4. Surface plasmon resonance (SPR) measurements

The binding kinetics of CacyBP/SIP to tubulin were monitored in real-time with a BIAcore 3000 instrument. Tubulin or BSA (as a control) was covalently linked to a Sensor Chip CM5 (carboxymethylated dextran surface) with the use of amine coupling chemistry according to the manufacturer's instructions. Tubulin or BSA solution in 10 mM sodium acetate, pH 4.5, was applied with a flow rate of 40 μl/min at 20 °C. The remaining free reactive groups on the chip were then inactivated by 1 M ethanolamine–HCl, pH 8.0, and tubulin or BSA not covalently bound was washed off with buffer A containing 10 mM phosphate buffer pH 7.0 and 100 mM NaCl. All experiments were performed at 37 °C. The volume of 230 μl of buffer A containing the CacyBP/SIP protein was simultaneously injected with a flow rate of 40 μl/min over a control surface with immobilized BSA and a surface with immobilized tubulin. After injection, buffer A with CacyBP/SIP was replaced by buffer A alone at a continuous flow rate of 40 μl/min. Surface regeneration was accomplished by injecting 5 μl of 0.1 M glycine pH 2.0. Each sensogram (time course of the SPR signal) was corrected for the response observed in the control flow cell unit. For each experiment different concentrations of CacyBP/SIP in buffer A were used. In the first experiment concentrations of CacyBP/SIP were: 7.13, 3.56, 1.43, 0.71, 0.36 μM and in the second one were: 9.71, 3.89, 0.78, 0.38, 0.19, 0.13 μM. Kinetic data obtained in two independent experiments were interpreted according to a 1:1 binding model using the BIAevaluation 4.1 SPR kinetic software. k_{on} was calculated from the association curves obtained for different CacyBP/SIP concentrations. Dissociation curves used to calculate k_{off} were obtained in the same experiments when the protein solution was replaced with the running buffer.

2.5. Cell transfection and co-immunoprecipitation assay

10-cm dishes of NB2a cells (70–80% confluent) were transfected with 8 μg of the expression plasmids: p3xFLAG-CMV-10-CacyBP/SIP or p3xFLAG-CMV-10-BAP using lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. After 24 h cells were washed twice with phosphate-buffered saline (PBS). Then cells were harvested and homogenized mechanically 30 times in immunoprecipitation buffer (IP) 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. Protein concentration was estimated by the Bradford procedure (BioRad) with BSA as a standard.

A 40 μl aliquot of 50% slurry of the anti-FLAG agarose beads (Sigma) equilibrated with IP buffer was used in each co-immunoprecipitation. 500 μg of