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Influence of S100A6 on CacyBP/SIP Phosphorylation and Elk-1 Transcriptional Activity in Neuroblastoma NB2a Cells

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ABSTRACT

In this work, we have found that casein kinase II (CKII) phosphorylates the CacyBP/SIP protein under in vitro conditions and have mapped the phosphorylation site to threonine 184. Moreover, we present evidence that S100A6, a CacyBP/SIP interacting protein, inhibits this phosphorylation in the presence of Ca^{2+}. CacyBP/SIP phosphorylation by CKII was also observed in neuroblastoma NB2a cells. Interestingly, we have found that the effect of DRB, a CKII inhibitor, on CacyBP/SIP phosphorylation state is similar to that of S100A6 overexpression. Phosphorylation at threonine 184 seems to have an effect on CacyBP/SIP phosphatase activity since the T184E phosphorylation mimic mutant overexpressed in NB2a cells has lower phosphatase activity toward p-ERK1/2 when compared to the non-phosphorylable T184A mutant or to the wild-type protein. In conclusion, our data suggest that S100A6 and Ca^{2+}, through inhibiting CacyBP/SIP phosphorylation on threonine 184, are important regulators of CacyBP/SIP phosphatase activity and of ERK1/2-Elk-1 signaling pathway. J. Cell. Biochem. 117: 126–131, 2016.

KEY WORDS: CacyBP/SIP; CASEIN KINASE II; ERK1/2; Elk-1; NB2a CELLS; S100A6

CacyBP/SIP was originally purified from Ehrlich ascities tumor cells as a ligand of S100A6 (calcyclin) [Filipek and Wojda, 1996; Filipek and Kuźnicki, 1998]. Up to now, many papers have been published describing CacyBP/SIP biochemical properties, its tissue and cell distribution and ligand binding (reviewed in Schneider and Filipek, 2011). It is known that CacyBP/SIP interacts with S100A6 and other members of the S100 family [Filipek et al., 2002] as well as with Siah-1 [Matsuzawa and Reed, 2001], Skp1 [Bhattacharya et al., 2005], tubulin [Schneider et al., 2007], actin [Schneider et al., 2010], tropomyosin [Jurewicz et al., 2013], ERK1/2 kinase [Kilanczyk et 2009; Kilanczyk et al., 2011], and tau [Wasik et al., 2013].

Interaction of CacyBP/SIP with tubulin, actin, and tropomyosin seems to be important in cytoskeleton organization during differentiation of the neuronal type of cells [Jastrzebska et al., 2000; Schneider et al., 2007] or during their degeneration [Filipek et al., 2008; Wasik et al., 2013]. Interestingly, the interaction of CacyBP/SIP with another target, ERK1/2 kinase, results in dephosphorylation of this kinase and, in consequence, in a decrease in phosphorylation/transcriptional activity of Elk-1 [Kilanczyk et al., 2009, 2011]. Recently, it has been shown that CacyBP/SIP is a novel regulator of CREB- and NFAT-driven transcription [Kilanczyk et al., 2015].

Regarding the interaction of CacyBP/SIP with calcium binding proteins of the S100 family it has been shown that CacyBP/SIP binds, in a Ca^{2+}-dependent way, not only S100A6 but also some other S100 proteins such as S100A1, S100A12, S100B, and S100P [Filipek et al., 2002]. Moreover, it has been found that the C-terminal part of CacyBP/SIP, comprising residues 178–229, is responsible for complex formation with these S100 proteins [Nowotny et al., 2000; Lee et al., 2008]. As to the role of the interaction between CacyBP/SIP and S100 proteins, there is some indirect evidence that S100A6, might negatively regulate the activity of Siah-1-CacyBP/SIP-Skp1 ubiquitin ligase, presumably in a Ca^{2+}-dependent manner [Lee et al., 2008; Mayer et al., 2008; Ning et al., 2012]. Since our theoretical analysis suggested that CacyBP/SIP might be phosphorylated by CKII on threonine 184 (T184), which is located close to the S100A6 binding site, in this work we analyzed whether CacyBP/SIP is phosphorylated by CKII in vitro and in neuroblastoma NB2a cells and whether S100A6 binding affects this phosphorylation. We also

Abbreviations: CKII, casein kinase II; DRB, 5,6-dichloro-1-beta-D-ribofuranosylbenzimidazole; ERK1/2, extracellular signal regulated kinases 1 and 2; Elk-1, transcription factor; PAGE, polyacrylamide gel electrophoresis; PARP, polyADP-ribose polymerase; PMA, phorbol 12-myristate 13-acetate; SDS, sodium dodecyl sulfate; 2D, two dimensional.

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examined the influence of T184 phosphorylation on CacyBP/SIP phosphatase activity toward ERK1/2 kinase.

MATERIALS AND METHODS

CELL-BASED EXPERIMENTS

Mouse neuroblastoma NB2a cells were cultured as described by Schneider et al. [2007]. Transfection of this cell line was carried out using lipofectamine2000 (Invitrogen) according to the manufacturer's protocol.

To monitor the level of p-ERK1/2, CacyBP/SIP-3xFLAG and its mutants, CacyBP/SIP-T184A-3xFLAG, and CacyBP/SIP-T184E-3xFLAG, were overexpressed in NB2a cells using 10 μg of the plasmid encoding an appropriate protein [Schneider et al., 2007] and cultured for 24 h. After that cells were stimulated with 6 nM PMA (PKC activator) for 1 h in order to elevate the level of p-ERK1/2. Then, cells were harvested and the nuclear fraction was obtained using the NE-PER extraction reagents (Pierce) according to the manufacturer's instruction.

In order to examine the influence of casein kinase II (CKII) inhibitor, DRB, or S100A6, on CacyBP/SIP forms exhibiting different isoelectric points in 2D electrophoresis, NB2a cells were incubated for 24 h in complete medium with DRB at 1 μM final concentration or transfected with plasmid encoding S100A6 [Spiechowicz et al., 2007; Wasik et al., 2013]. Cells harvested after DRB treatment or 24 h after transfection were incubated in RIPA buffer containing 20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.5% NP-40, 1% Triton X-100, supplemented with protease and phosphatase inhibitors (Roche) for 30 min on ice. The lysate was centrifuged at 20,000*g* for 20 min at RT and protein (150 μg) from the supernatant fraction was subjected to 2D electrophoresis and then to Western blotting.

To assess the Elk-1 transcriptional activity in NB2a cells the PATH-DETECT trans-reporting system (Stratagene) containing a path specific fusion trans-activator plasmid (pFA2-ELK1) and a reporter plasmid (PFR-Luc) controlling the expression of the luciferase gene were used. NB2a cells were transfected with these plasmids together with pcDNA3.1-HA-ERK2 and pcDNA3.1-CacyBP/SIP [Schneider et al., 2007] or co-transfected with pcDNA3.1-S100A6 plasmid when needed [Spiechowicz et al., 2007; Wasik et al., 2013]. As an internal control reporter, the pRL-SV40 plasmid (Promega) was used. Transfection was performed using lipofectamine2000 (Invitrogen) and luciferase activity was assessed after 24 h using the dual-luciferase reporter assay system (Promega) and a TD-20/20 luminometer (Turner Designs).

PREPARATION OF pCMV3xFLAG-CacyBP/SIP-T184A/T184E EXPRESSION PLASMIDS

The plasmids encoding FLAG-CacyBP/SIP mutants (T184A or T184E) were generated employing site-directed mutagenesis with pCMV3xFLAG-CacyBP/SIP as a template. In the first case the threonine residue at position 184 was changed to alanine in a PCR reaction using *Pfu* polymerase and the following primers: forward: CTTCCTACGACGCGGAGGCAGACCC and reverse: CTAGGGTCTGCCTCCGCGTCGTAGG. In order to obtain a mutant in which threonine at position 184 was changed to glutamic acid three steps were performed using three pairs of primers: I-forward: CTTCCTACGACGCGGAGGCAGACCC and reverse: CTAGGGTCTGCCTCCGCGTCGTAGG, II-forward: CTTCCTACGACGAGGAGGCAGACCC and reverse: CTAGGGTCTGCCTCCTCGTCGTAGG, III-forward: CTTCCTACGACGACGAGGCAGACCC and reverse: CTAGGGTCTGCCTCGTCGTCGTAGG. To remove the template DNA the PCR product was treated with *DpnI* endonuclease. The correctness of the pCMV3xFLAG-CacyBP/SIP(T184A or T184E) constructs, after *DpnI* treatment, were verified by DNA sequencing.

PROTEIN PURIFICATION AND AUTORADIOGRAPHY

Recombinant CacyBP/SIP was purified as described by Schneider et al. [2007]. S100A6 was purified as described by Slomnicki et al. [2009] while S100A4 and S100B were purchased from R&D Systems and Sigma company, respectively. Casein kinase II (CKII) was purchased (Enzo Life Sciences). Briefly, 500 ng of CKII kinase was preincubated with 10 μl of buffer containing 20 mM HEPES pH 7.5, 10 mM $MgCl_2$, 50 mM NaCl, 1 mM DTT, and 0.2 mM ATP for 20 min at 30°C. Then, 1 μg of CacyBP/SIP and 1 μg of a given S100 protein, preincubated for 15 min in the same buffer with either 1 mM $CaCl_2$, or 2 mM EGTA, were added together with 1 μl [γ-^{32}P] ATP (Hartman Analytic, 9,25 MBq in 25 μl). The reaction was carried out for 30 min at 30°C and stopped by addition of SDS sample buffer and boiling. The proteins were separated in SDS–PAGE and then gels were dried and exposed to a photographic film (Kodak).

THEORETICAL ANALYSIS OF CacyBP/SIP SEQUENCE AND MASS SPECTROMETRY

The theoretical analysis of putative phosphorylation sites in the CacyBP/SIP sequence was performed using NetPhos 2.0 server (www.cbs.dtu.dk/services/NetPhos/). In order to identify kinase(s) potentially involved in CacyBP/SIP phosphorylation a group-based prediction system (http://gps.biocuckoo.org/) was applied.

To confirm that threonine 184 in CacyBP/SIP might be phosphorylated by CKII and that S100A6 can interfere with this process the phosphorylation of CacyBP/SIP was performed as for autoradiography with the exception that [γ-^{32}P] ATP was replaced by cold ATP. The bands corresponding to CacyBP/SIP were cut off from the SDS gel and analyzed by mass spectrometry. ESI-MS peptide sequencing was performed in the Institute of Biochemistry and Biophysics, Warsaw, Poland.

2D ELECTROPHORESIS, SDS–PAGE AND WESTERN BLOT

For isoelectrofocusing, NB2a cell lysate (150 μg protein), obtained as described above in the presence of phosphatase inhibitors (PhosSTOP, Roche), was applied on linear pH gradient (pH 3–10) strips (BioRad). The isoelectrofocused proteins were then separated by SDS–PAGE followed by Western blot.

Electrophoresis with gels containing 10% (w/v) polyacrylamide and 0.1% SDS was performed by the method of Laemmli [1970]. Proteins were then transferred onto nitrocellulose and identified using appropriate primary antibodies: rabbit polyclonal anti-p-ERK1/2 (Cell Signaling) diluted 1:1000, mouse monoclonal anti-CacyBP/SIP (Abcam) diluted 1:1000, mouse monoclonal anti-FLAG (Sigma) diluted 1:5000. Equal protein loading was assessed by staining with anti-PARP antibody (Cell Signaling) diluted 1:2000.

After washing with TBS–T buffer (50 mM Tris pH 7.5, 200 mM NaCl, 0.05% Tween-20) blots were allowed to react with secondary goat anti-mouse IgG (1:10000; Jackson Immunoresearch Laboratories) or goat anti-rabbit IgG (1:10000; MP Biomedicals) antibodies. After three washes with the TBS–T buffer and two washes with the TBS buffer (50 mM Tris pH 7.5, 200 mM NaCl) blots were developed with the ECL chemiluminescence kit (Amersham Biosciences) followed by exposure against a RETINA X-ray film. The intensities of protein bands from Western blots were quantified using the Ingenius Bio-Imagining system (Syngene) and the Gene Tools software using FLAG as a reference protein. Statistical data analysis was performed using Student's *t* test.

RESULTS

PHOSPHORYLATION OF CacyBP/SIP BY CASEIN KINASE II (CKII)

To establish the potential phosphorylation sites in CacyBP/SIP sequence we have performed theoretical analysis using the NetPhos 2.0 server (www.cbs.dtu.dk/services/NetPhos/). This analysis indicated not only potential PKC phosphorylation sites [Kilanczyk et al., 2012] but also one potential phosphorylation site for CKII, namely threonine 184. Threonine 184 is located in a close distance to the CacyBP/SIP fragment interacting in a Ca^{2+}-dependent manner with S100A6 [Nowotny et al., 2000; Lee et al., 2008]. Thus, we have checked experimentally the phosphorylation of purified, recombinant CacyBP/SIP by CKII and the influence of S100A6 binding on this modification.

By applying autoradiography, we have found that phosphorylation of CacyBP/SIP by CKII was effective when the protein was preincubated with S100A6 in the presence of EGTA but not in the presence of Ca^{2+} (Fig. 1). To check the specificity of S100A6 binding on inhibition of CacyBP/SIP phosphorylation by CKII, we included two other proteins from the S100 family, S100A4 and S100B. S100A4 does not interact while S100B interacts in a Ca^{2+}- dependent manner with CacyBP/SIP [Filipek et al., 2002]. As expected, S100A4 does not influence CacyBP/SIP phosphorylation depending on Ca^{2+} while the effect of S100B is, to some extent, similar to that of S100A6. Altogether, the data suggest that CacyBP/SIP is phosphorylated by CKII and that in the presence of Ca^{2+}, the S100A6 protein inhibits this modification. As a control, phosphorylation of CacyBP/SIP by CKII was performed in the presence or absence of Ca^{2+} without any S100 protein present. As it can be seen in Figure 1, Ca^{2+} has no effect on CacyBP/SIP phosphorylation by CKII in the absence of S100 proteins, which indicates that Ca^{2+}-dependent interaction of S100A6 (and of some other S100 proteins such as S100B) with CacyBP/SIP plays an important role in this modification.

In order to experimentally identify the residue(s) phosphorylated by CKII, CacyBP/SIP was phosphorylated in the presence of cold ATP and then the corresponding band was cut off from the gel and analyzed by mass spectrometry. In order to confirm that phosphorylation depends on S100A6 and Ca^{2+} concentration, the reaction was performed in the presence of S100A6 and in the presence or absence of Ca^{2+} (presence of EGTA). Mass spectrometry analysis revealed that threonine 184 was unequivocally phosphorylated in both samples, however, when the normalized signal intensity of the peptide containing threonine 184 was compared between Ca^{2+} and EGTA samples, the average signal intensity corresponding to phosphorylated threonine T184 was 13.5 times higher in EGTA samples.

PHOSPHORYLATION OF CacyBP/SIP BY CASEIN KINASE II (CKII) IN NEUROBLASTOMA NB2a

In order to check whether CacyBP/SIP might be phosphorylated by CKII in NB2a cells, we have compared the pattern of spots representing CacyBP/SIP forms after 2D electrophoresis in cell lysates obtained from control and DRB (CKII inhibitor) treated cells (Fig. 2). We have found that the ratio of two major CacyBP/SIP forms,

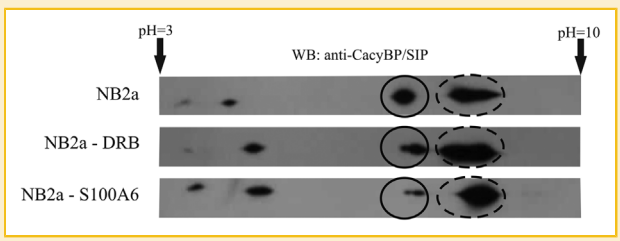

Fig. 2. Influence of DRB and S100A6 on the pattern of CacyBP/SIP forms in 2D electrophoresis. Upper panel shows spots representing endogenous CacyBP/SIP forms present in the total lysate obtained from NB2a cells. Middle panel shows CacyBP/SIP forms after treatment of NB2a cells with specific CKII inhibitor, DRB. Lower panel shows CacyBP/SIP forms in NB2a cells after overexpression of S100A6. 150 μg of protein were applied on the strips. A representative Western blot experiment, out of three performed, is shown.

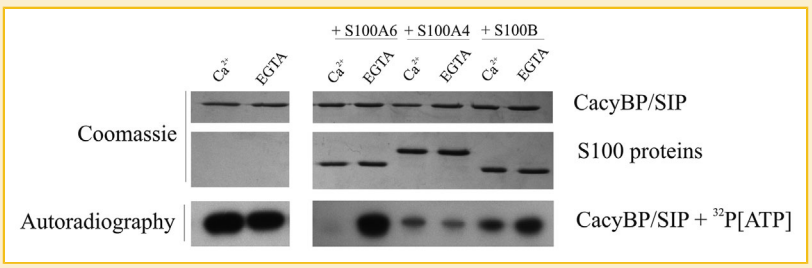

Fig. 1. Phosphorylation of CacyBP/SIP in vitro by casein kinase II with [γ-^{32}P]ATP. Upper panel shows Coomassie stained gels and the lower panel shows autoradiogram. A representative experiment, out of three performed, is shown.

having different isoelectric points, pI ~ 7.0 (marked by circles) and pI ~ 8.0 (marked by dashed circles), changes after DRB treatment. After DRB treatment the amount of the more basic form (pI ~ 8.0) was higher than in control at the expense of the form with pI ~ 7.0, suggesting that the latter one may represent CKII phosphorylated CacyBP/SIP. Similarly, a larger spot corresponding to the CacyBP/SIP form with pI ~ 8.0, was obtained when S100A6 was overexpressed, indicating that S100A6 inhibited phosphorylation of CacyBP/SIP similarly as DRB did. The ratio of the pI 8.0 versus the pI 7.0 CacyBP/SIP forms changed from 1.7 in control NB2a cells to 4.8 and 8.1 in DRB treated and S100A6 overexpressing cells, respectively.

INFLUENCE OF PHOSPHORYLATION OF THREONINE 184 IN CacyBP/SIP ON p-ERK1/2 LEVEL AND Elk-1 ACTIVITY

In order to check whether phosphorylation of threonine 184 has an effect on the level of p-ERK1/2, plasmids encoding FLAG-tagged CacyBP/SIP mutants in which threonine 184 was replaced by alanine (CacyBP/SIP-T184A) or by glutamic acid (CacyBP/SIP-T184E), were constructed. NB2a cells were transfected with these plasmids and the level of p-ERK1/2 in nuclear fractions was analyzed. As it could be seen in Figure 3 (A and B), the level of p-ERK1/2 in the nuclear fraction obtained from cells transfected with plasmid encoding the T184E CacyBP/SIP phosphorylation mimic mutant, was higher by about 55% (155.28% +/− 26.13%) than in the nuclear fraction obtained from cells transfected with plasmid encoding wild type CacyBP/SIP or its T184A non-phosphorylable mutant. This result clearly shows that dephosphorylation of threonine 184 increases, while its phosphorylation decreases, the CacyBP/SIP phosphatase activity toward ERK1/2. Thus, the higher activity of the CacyBP/SIP non-phosphorylable form (T184A mutant) corresponds to the lower level of CacyBP/SIP phosphorylation detected in the presence of S100A6 and Ca^{2+} (Fig. 1).

As it has been already shown, a decreased level of p-ERK1/2 correlates with a decreased level/transcriptional activity of p-Elk-1 [Kilanczyk et al., 2009]. To check whether S100A6 affects the transcriptional activity of Elk-1 we performed a luciferase assay using a reporter vector carrying an artificial promoter controlled by the GAL4/Elk-1 fusion protein. We have found that overexpression of S100A6 diminished the luciferase signal, that is, Elk-1 activity (Fig. 4). This effect might result from stimulation of CacyBP/SIP phosphatase activity by S100A6 due to blocking phosphorylation on T184.

DISCUSSION

Post-translational modifications, among them phosphorylation, are known to modulate protein function [Han and Martinage, 1992]. Recently, it was found that CacyBP/SIP phosphatase is phosphorylated by PKC on serine 22 and threonine 23 and that phosphorylation of these residues increases the phosphatase activity toward ERK1/2

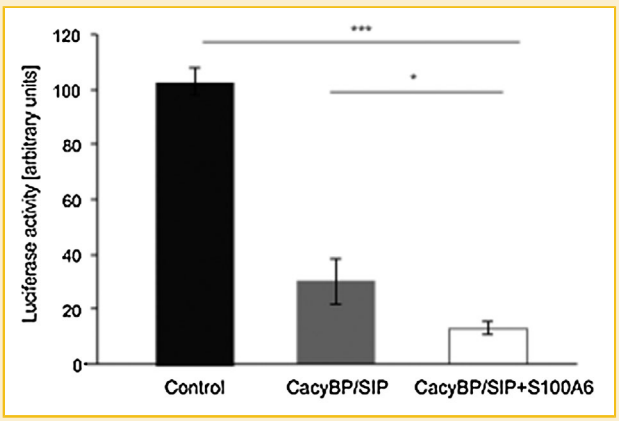

Fig. 4. Effect of CacyBP/SIP, in the presence or absence of S100A6, on Elk-1 transcriptional activity. Activity was assessed by luciferase assay. Black bar represents the Elk-1 activity in cells transfected with pcDNA3.1-HA-ERK2 alone (control), white bar shows Elk-1 activity in cells co-transfected with pcDNA3.1-CacyBP/SIP and the dotted bar represents Elk-1 activity in cells co-transfected with pcDNA3.1-CacyBP/SIP and pcDNA3.1-S100A6. Results from three independent experiments are presented as a mean ± SD. $^{*}P \leq 0.05$; $^{***}P \leq 0.001$.

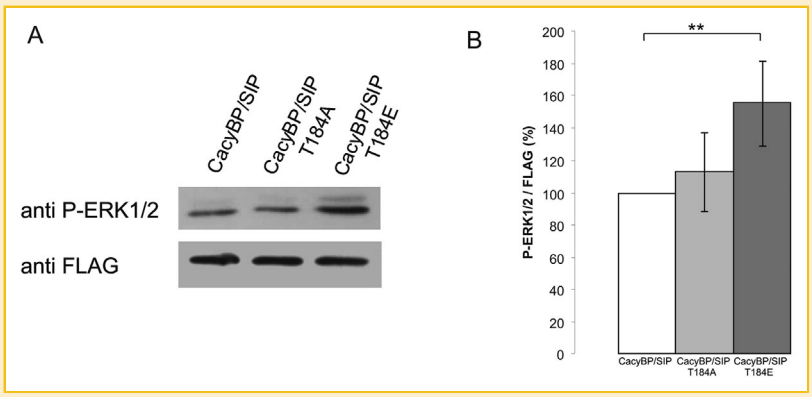

Fig. 3. Influence of threonine 184 mutation on CacyBP/SIP phosphatase activity toward p-ERK1/2. A: Western blot showing the level of phosphorylated ERK1/2 (p-ERK1/2) in the nuclear fraction of NB2a cells overexpressing FLAG-tagged CacyBP/SIP or its mutants. B: Statistical analysis of data from three independent experiments presented as the mean ± SD; $^{**}P \leq 0.01$.

[Kilanczyk et al., 2012; Topolska-Woś et al., 2015]. In this work, we have analyzed CacyBP/SIP phosphorylation by CKII since theoretical analysis indicated the presence of one potential phosphorylation site for that kinase, namely threonine 184. Threonine 184 is located in close proximity to the fragment of CacyBP/SIP which interacts in a Ca^{2+}-dependent manner with S100A6 [Nowotny et al., 2000; Lee et al., 2008]. Thus, we have experimentally checked phosphorylation of purified, recombinant CacyBP/SIP by CKII and the influence of S100A6 on this modification. We have found that CKII phosphorylates CacyBP/SIP under in vitro conditions and that S100A6, in the presence of Ca^{2+}, inhibits this phosphorylation. When two other S100 proteins, S100A4 and S100B were used, it appeared, as expected, that S100A4 does not have any effect on CacyBP/SIP phosphorylation depending on Ca^{2+} while S100B does. Interestingly, in the case of S100A4, CacyBP/SIP phosphorylation was inhibited (both in Ca^{2+} and EGTA) when compared to S100A6 and S100B. It suggests that S100A4 may bind to some extent to CacyBP/SIP in Ca^{2+}-independent way or that S100A4 may have an effect on CKII kinase activity. When a specific CKII inhibitor, DRB, was present or S100A6 was overexpressed in NB2a cells, the pattern of CacyBP/SIP spots in 2D electrophoresis was changed. Namely, the level of the more basic CacyBP/SIP form increased at the expense of the second major spot as compared to control cells. Moreover, we have also found that the T184E CacyBP/SIP mutant, which mimics the phosphorylation of CacyBP/SIP on T184, has lower phosphatase activity toward p-ERK1/2 when compared to the T184A CacyBP/SIP non-phosphorylable mutant or wild type CacyBP/SIP. The comparable activity of T184A CacyBP/SIP mutant and of the wild type protein may indicate that the latter is not highly phosphorylated in NB2a cells. On the other hand, lower activity of the T184E mutant is especially interesting in the light of the results showing that CacyBP/SIP phosphorylation at serine 22 and threonine 23 resulted in higher phosphatase activity toward ERK1/2 [Kilanczyk et al., 2012; Topolska-Woś et al., 2015]. Together, the results concerning CacyBP/SIP phosphorylation suggest that its activity depends on the kinases and the sites involved. This in turn implicates that the activity might be differentially regulated by various signaling cascades. The Ca^{2+}-dependent regulation of CacyBP/SIP phosphatase activity by S100A6 might be of physiological importance when the intracellular level of S100A6 and Ca^{2+} is increased, for instance in tumor cells (reviewed in Schneider and Filipek, 2011).

Altogether, results described in the present work show for the first time that CacyBP/SIP is phosphorylated on threonine 184 by CKII and that this phosphorylation is inhibited by S100A6 in a Ca^{2+}-dependent manner. This finding is important since up to now little was known regarding the regulation of CacyBP/SIP function by the originally identified target, S100A6. Our data clearly indicate that phosphorylation/dephosphorylation of threonine 184 and regulation of this modification by S100A6 is important for CacyBP/SIP phosphatase activity and for the ERK1/2- Elk-1 signaling pathway.

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