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CacyBP/SIP binds ERK1/2 and affects transcriptional activity of Elk-1

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ABSTRACT

In this work we showed for the first time that mouse CacyBP/SIP interacts with extracellular signal regulated kinases 1 and 2 (ERK1/2). We also established that a calcium binding protein, S100A6, competes for this interaction. Moreover, the E217K mutant of CacyBP/SIP does not bind significantly to ERK1/2 although it retains the ability to interact with S100A6. Molecular modeling shows that the E217K mutation in the 189–219 CacyBP/SIP fragment markedly changes its electrostatic potential, suggesting that the binding with ERK1/2 might have an electrostatic character. We also demonstrate that CacyBP/SIP-ERK1/2 interaction inhibits phosphorylation of the Elk-1 transcription factor in vitro and in the nuclear fraction of NB2a cells. Altogether, our data suggest that the binding of CacyBP/SIP with ERK1/2 might regulate Elk-1 phosphorylation/transcriptional activity and that S100A6 might further modulate this effect via Ca^{2+}-dependent interaction with CacyBP/SIP and competition with ERK1/2.

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CacyBP/SIP was originally discovered as a S100A6 (calcyclin) binding partner [1]. Later, it was found that the human ortholog of CacyBP/SIP interacts with Siah-1 and Skp1 proteins and that it might play a role in ubiquitination of β-catenin [2]. Shortly after CacyBP/SIP was discovered, Pircher et al. [3] showed that the level of CacyBP/SIP mRNA is increased during differentiation of erythroid cells. Later, it has been reported that CacyBP/SIP could be implicated in the development of thymocytes [4], in endometrial events during pregnancy in mouse [5,6] and in the differentiation of rat neonatal cardiomyocytes [7]. Studies performed by Wu et al. [8] on human neuroblastoma SH-SY5Y cells and our work done on mouse neuroblastoma NB2a cells [9,10] suggest that CacyBP/SIP might be involved in differentiation of neuronal cells. It is known that differentiation is regulated by many cofactors, among them are the extracellular signal regulated kinases 1 and 2 (ERK1/2) [11]. During cell differentiation, ERK1/2 become phosphorylated and translocated to the nucleus, where they can activate transcription factors such as Elk-1 [12].

Since CacyBP/SIP and ERK1/2 are involved in cell differentiation in this work we wanted to establish if CacyBP/SIP may affect the activity of ERK1/2. For that we checked whether CacyBP/SIP binds to ERK1/2 and if the interaction between CacyBP/SIP and ERK1/2 has an impact on the phosphorylation of Elk-1. We also performed experiments in order to assess whether the E217K mutant of CacyBP/SIP interacts with ERK1/2 and if another target of CacyBP/SIP, S100A6, competes with ERK1/2 for this binding. Our results show that CacyBP/SIP binds to ERK1/2 and through this interaction regulates the phosphorylation of Elk-1 in vitro and in the nuclear fraction of NB2a cells.

Materials and methods

Cell—based experiments, plasmid construction and protein purification. Mouse neuroblastoma NB2a cells were cultured and differentiated as described by Schneider et al. [10]. The nuclear fraction from NB2a cells was obtained using the NE-PER nuclear and cytoplasmic extraction reagents (Pierce) according to manufacturer's instruction. Samples containing 100 μg of proteins from nuclear fractions were analyzed by SDS–PAGE and then by Western blotting. The intensities of protein bands from Western blot of nuclear fractions were quantified using the Ingenius Bio-Imaging (Syngene) and the Gene Tools software with PARP as a reference protein.

Transfection of NB2a cells was carried out using Lipofectamine2000 (Invitrogen) according to the manufacturer's protocol. Cells were transfected with 10 μg of p3xFLAG-CMV-10-CacyBP/

Abbreviations: BSA, bovine serum albumin; DMEM, Dulbecco's Modified Eagle's Medium; DTT, dithiothreitol; EDTA, ethylene diamine tetraacetic acid; ERK1/2, extracellular signal regulated kinases 1 and 2; MEK, MAPK/ERK kinases; PAGE, polyacrylamide gel electrophoresis; PARP, PolyADP-ribose polymerase; PBS, phosphate buffered saline; PMA, phorbol myristate acetate; SDS, sodium dodecyl sulfate; TMB, tetramethylbenzidine.

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SIP, and after 24 h cells on one 10 cm dish were differentiated for 24 h using palmitoylcarnitine and cells on another dish were stimulated for 1 h with PMA at a final concentration of 6 nM. After that protein extracts were prepared and immunoprecipitation was performed as described by Schneider et al. [10].

Construction of plasmids used for cell transfection as well as for recombinant protein purification are described in Supplementary material. The E217K CacyBP/SIP mutant and ERK2 were purified in the same way as wild type CacyBP/SIP [13]. The S100A6 protein was purified as described by Slomnicki et al. [14].

SDS–PAGE and Western blotting. Gel electrophoresis with 10% (w/v) polyacrylamide containing 0.1% SDS was performed by the method of Laemmli [15]. Proteins were transferred electrophoretically onto nitrocellulose and identified using appropriate primary antibodies: rabbit anti-CacyBP/SIP affinity purified polyclonal antibody (1:100), mouse anti-FLAG M2 antibody (1:3000) (Sigma), polyclonal anti-P-ERK1/2 (1:1000) (Cell Signaling), polyclonal anti-ERK1/2 (1:1000) (Cell Signaling), polyclonal anti-P-Elk-1 (1:1000) (Cell Signaling), or monoclonal anti-PARP antibody (Alexis Biochemicals) at a 1:500 dilution. 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 antibodies either: goat anti-mouse IgG (1:10,000) (Jackson Immunoresearch Laboratories) or goat anti-rabbit IgG (1:5000) (MP Biomedicals) conjugated to horseradish peroxidase. 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 exposition against an X-ray film.

ELISA. CacyBP/SIP or its E217K mutant or BSA (400 ng/well) were immobilized onto a 96-well microtiter plate in 50 μl of coating buffer (25 mM HEPES, pH 7.6, 100 mM KCl). After 1.5 h incubation at room temperature the solution was removed and wells were washed four times with washing buffer (25 mM HEPES, pH 7.6, 100 mM KCl, 2 mg/ml BSA). The last wash lasted 1 h. Next, increasing amounts of P-ERK1/2 (in activity units, ELISA kit, Sigma) or purified recombinant ERK2 or S100A6 were added and after incubation for 1 h at room temperature the solution was removed and the plates were washed three times with the washing buffer. Then, rabbit polyclonal antibodies against P-ERK1/2 at a 1:5000 dilution (Sigma), against ERK1/2 at a 1:5000 dilution (Cell Signaling) or against S100A6 (affinity purified) at a 1:100 dilution [16] were added and the plates were incubated for 2 h at room temperature. After that, the plates were washed three times and the anti-rabbit IgG-horseradish peroxidase conjugated with secondary antibody (Sigma) at a 1:8000 dilution was added to each well for 1 h. The plates were washed three times and the analysis of bound antibodies was performed by colorimetric detection with TMB reagent. The reaction was stopped with 100 μl of 1 M sulfuric acid and the absorbance at 450 nm was measured using the Thermo Labsystem microplate reader.

In the competition assay, CacyBP/SIP (400 ng/well) was immobilized and 200 ng of ERK2 were added together with an increasing amount of S100A6 (the molar ratio of S100A6 dimer to ERK2 was 0.125:1, 0.25:1, 0.5:1, 1:1, 2:1 and 5:1, respectively).

Molecular modeling. The structure of CacyBP/SIP fragment (residues 189–219) containing E217 was taken from the NMR structure of the complex of this protein with the S100A6 dimer (Protein Data Bank identification code 2JTT) [17]. Twenty NMR structures were analyzed and the most representative one was taken for calculations. The electrostatic potential of wild type protein and the E217K mutant was calculated by the Yasara program version 8.9 [18]. The structure of the E217K mutant was prepared by replacement of the E217 residue and subsequent energy minimization of the fixed CacyBP/SIP fragment apart from residue 217, the movement of which was not restrained. Acetyl and *N*-methyl groups were added to both ends of the CacyBP/SIP fragment examined to eliminate unwanted effects of charged ends on the overall electrostatic potential.

In vitro kinase assay and luciferase reporter system. Non-radioactive ERK1/2 kinase assay was performed according to the manufacturer's protocol (Cell Signaling) using a protein lysate from NB2a cells.

To assess the Elk-1 transcriptional activity in NB2a cells we used 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. Cells were transfected with these plasmids together with pcDNA3-HA-ERK2 and pcDNA-CacyBP/SIP (described by Schneider et al. [10]) or pEGFP-CacyBP/SIP(E217K). As an internal control reporter, pRL-SV40 plasmid (Promega) was used. Transfection was performed using Lipofectamin2000 (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).

Results

Co-immunoprecipitation of ERK1/2 with CacyBP/SIP

To check whether CacyBP/SIP interacts with ERK1/2 we performed co-immunoprecipitation assay in which we used extracts from differentiated, PMA-treated and undifferentiated NB2a cells transfected with p3xFLAG-CMV-10-CacyBP/SIP. Cell extracts were applied on agarose beads with coupled anti-FLAG antibodies. As shown in Fig. 1, ERK1/2 co-immunoprecipitate with CacyBP/SIP-FLAG as detected by the anti-P-ERK1/2 antibody (upper panel) or by the anti-ERK1/2 antibody (middle panel). The most intensive bands are seen in the case of differentiated and PMA-treated cells which express higher amount of ERK1/2 in comparison to undifferentiated ones. The staining with anti-FLAG antibodies shows the amount of protein extracts used for each immunoprecipitation (Fig. 1, lower panel).

Interaction of ERK1/2 and S100A6 with CacyBP/SIP and its E217K mutant

To confirm the interaction between ERK1/2 and CacyBP/SIP we performed an ELISA. For that purpose CacyBP/SIP or BSA (a control protein) were immobilized onto a 96-well microtiter plate and then increasing amounts of ERK1/2 (ELISA kit, Sigma) were applied. Bound ERK1/2 were allowed to react with anti-P-ERK1/2 antibodies and the analysis was performed by colorimetric detection. As it can be seen in Fig. 2A, CacyBP/SIP interacts with ERK1/2, which suggests that the binding is direct. We also checked the interaction of the recombinant ERK2 with CacyBP/SIP and with its E217K mutant. The mouse E217K mutant of CacyBP/SIP was designed on the

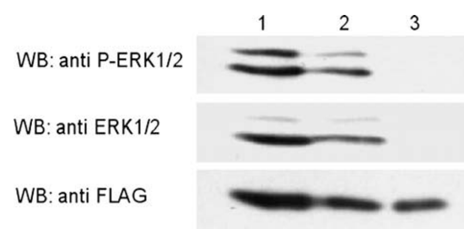

Fig. 1. Western blot showing co-immunoprecipitation of ERK1/2 with CacyBP/SIP-3xFLAG. Cells were transfected with plasmid encoding 3xFLAG-CacyBP/SIP and then treated with PMA (lane 1) or palmitoylocarnitine (lane 2) or untreated (lane 3). Blots were developed with: anti-P-ERK1/2 antibody (upper panel), anti-ERK1/2 antibody (middle panel) and anti-FLAG antibody (lower panel).

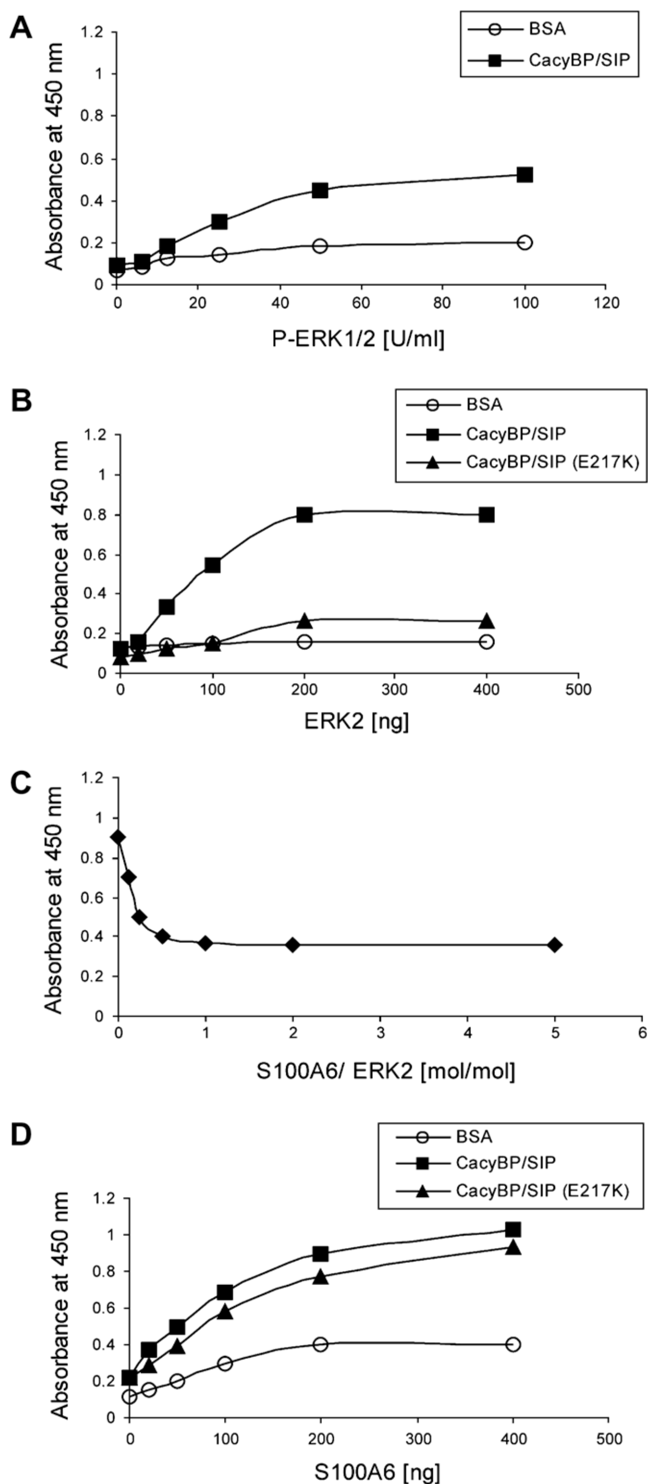

Fig. 2. ELISA. (A) Interaction of CacyBP/SIP and P-ERK1/2 estimated by an antibody against P-ERK1/2 and (B) interaction of CacyBP/SIP and its E217K mutant with ERK2 estimated by an antibody against ERK1/2. (C) Competition between the binding of ERK2 and S100A6 to immobilized CacyBP/SIP estimated using an anti-ERK1/2 antibody. (D) Interaction between S100A6 and CacyBP/SIP or its E217K mutant estimated using an anti-S100A6 polyclonal antibody. About 400 ng/well of CacyBP/SIP, its E217K mutant or BSA were immobilized onto ELISA plate wells. In each case a representative experiment out of three is shown.

basis of the E216K mutant of human CacyBP/SIP which was shown to largely ablate the effects of Siah-1 overexpression on β-catenin level [2]. As it can be seen in Fig. 2B, the CacyBP/SIP protein interacts with ERK2 while the binding between the E217K mutant and ERK2 appears to be very weak. Since the E217K mutation is located within the S100A6 binding domain (residues 189–219) [17,19], we performed an experiment in order to check the competition between S100A6 and ERK2 for the binding to CacyBP/SIP. The results obtained in competition ELISA show that S100A6 indeed competes with ERK2 for binding to CacyBP/SIP (Fig. 2C). This confirms that ERK2 interacts with the S100A6 binding domain of CacyBP/SIP and suggests that a calcium binding protein, S100A6, might regulate the ERK1/2–CacyBP/SIP interaction under the in vivo conditions. Since the E217K mutant of CacyBP/SIP almost did not bind to ERK2 we checked its binding to S100A6. Interestingly, we found by ELISA, that the interaction between the E217K mutant and S100A6 was preserved (Fig. 2D). The latter interaction was confirmed by cross-linking experiment (Fig. 1S, supplementary material).

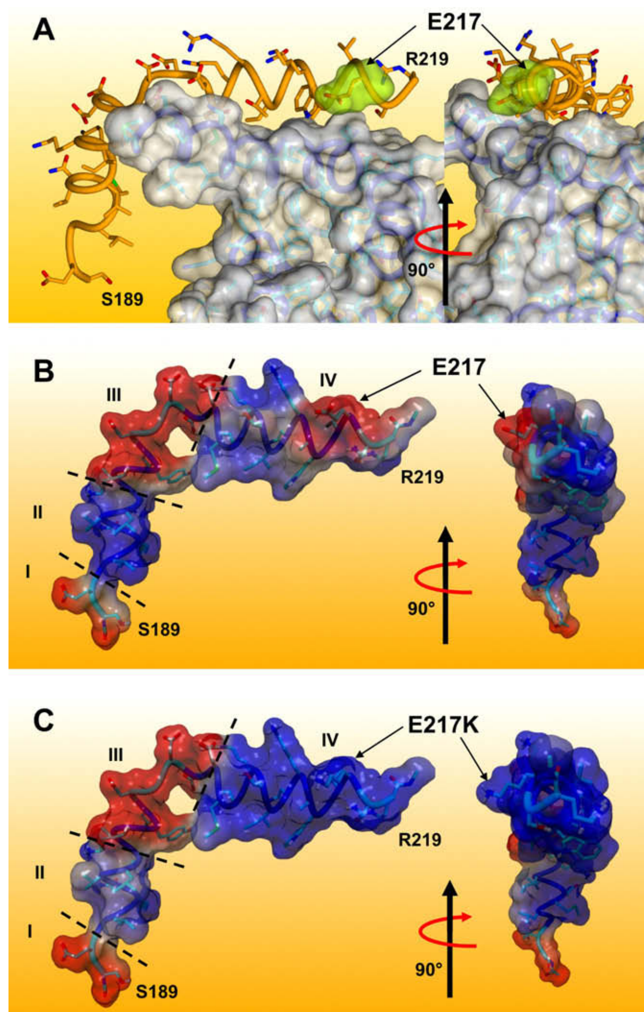

Fig. 3. Molecular modeling. (A) Structure of a complex formed between the CacyBP/SIP fragment (residues 189–219) and S100A6 (NMR structure code 2JTT from Protein Data Bank). CacyBP/SIP is shown as a wire whereas S100A6 as a transparent white surface. Only one S100A6 protomer is shown to make the picture clear. Residue E217 of CacyBP/SIP is indicated by a yellow–green transparent surface. (B) Molecular surface of the 189–219 fragment of the wild type and (C) of the E217K mutant of CacyBP/SIP mapped with electrostatic potential. The following coloring scale was used: negative potential in red, neutral in white and positive in blue. Areas I, II, III and IV are separated by dashed lines. The respective structures after 90° rotation are shown on the right side. (For interpretation of color mentioned in this figure the reader is referred to the web version of the article.)

Changes in electrostatic potential in CacyBP/SIP molecule induced by E217K mutation

To explain differential reactivity of the E217K mutant of CacyBP/SIP towards ERK1/2 and S100A6 we analyzed the structure of the S100A6 binding domain (residues 189–219) in CacyBP/SIP described by Lee et al. [17] and applied the molecular modeling. As it can be seen on Fig. 3A, the residue E217 of CacyBP/SIP is located closely to the surface of the S100A6 protein. This residue sticks out of the helix and does not bind to any adjacent positively charged residues in CacyBP/SIP. As it can be seen on Fig. 3B, the electrostatic potential of the examined fragment forms a characteristic pattern of alternative areas (I–IV). The first area (I) consists of two residues only, 189–190, and is negatively charged because of E190. The second area (II), comprising residues 191–198, is positively charged because of residues K197 and K198. The third area (III), containing residues 199–206, is strongly negatively charged because of residues E210, D202, D204, D205 and D206. The fourth area (IV), comprising residues 207–219, contains strongly positively charged residues K208, R209, K213 and R219, but because of the presence of E217 its overall electrostatic potential is close to neutral (indicated by white color in Fig. 3B) except for a small negatively charged spot originating from E217. After mutation of E217 to K, the electrostatic potential of this area converts into a strongly positive one (Fig. 3C), whereas that of area II changes to neutral values. The structures viewed after 90° rotation also show large changes in electrostatic properties of the analyzed CacyBP/SIP fragment.

The effect of CacyBP/SIP on phosphorylation and transcriptional activity of Elk-1

Since CacyBP/SIP interacts with ERK1/2 we checked its influence on ERK1/2 activity by assessing the level of phosphorylated Elk-1 (P-Elk-1). We performed a kinase assay using a non-radioactive ERK1/2 kit (Cell Signaling). As it can be seen in Fig. 4A, CacyBP/SIP inhibits phosphorylation of Elk-1. Densitometric analysis of western blots obtained from three independent experiments revealed about 60% decline in the phosphorylation level. As expected, the E217K mutant of CacyBP/SIP had no effect on this phosphorylation.

To confirm these results we performed another assay in which the transcriptional activity of Elk-1 was assessed by means of a reporter vector carrying the luciferase gene under an artificial promoter controlled by a GAL4/Elk-1 fusion protein. We found that overexpression of CacyBP/SIP and ERK2 diminished the luciferase signal suggesting the influence of CacyBP/SIP on Elk-1 transcriptional activity which depends on its phosphorylation by ERK1/2

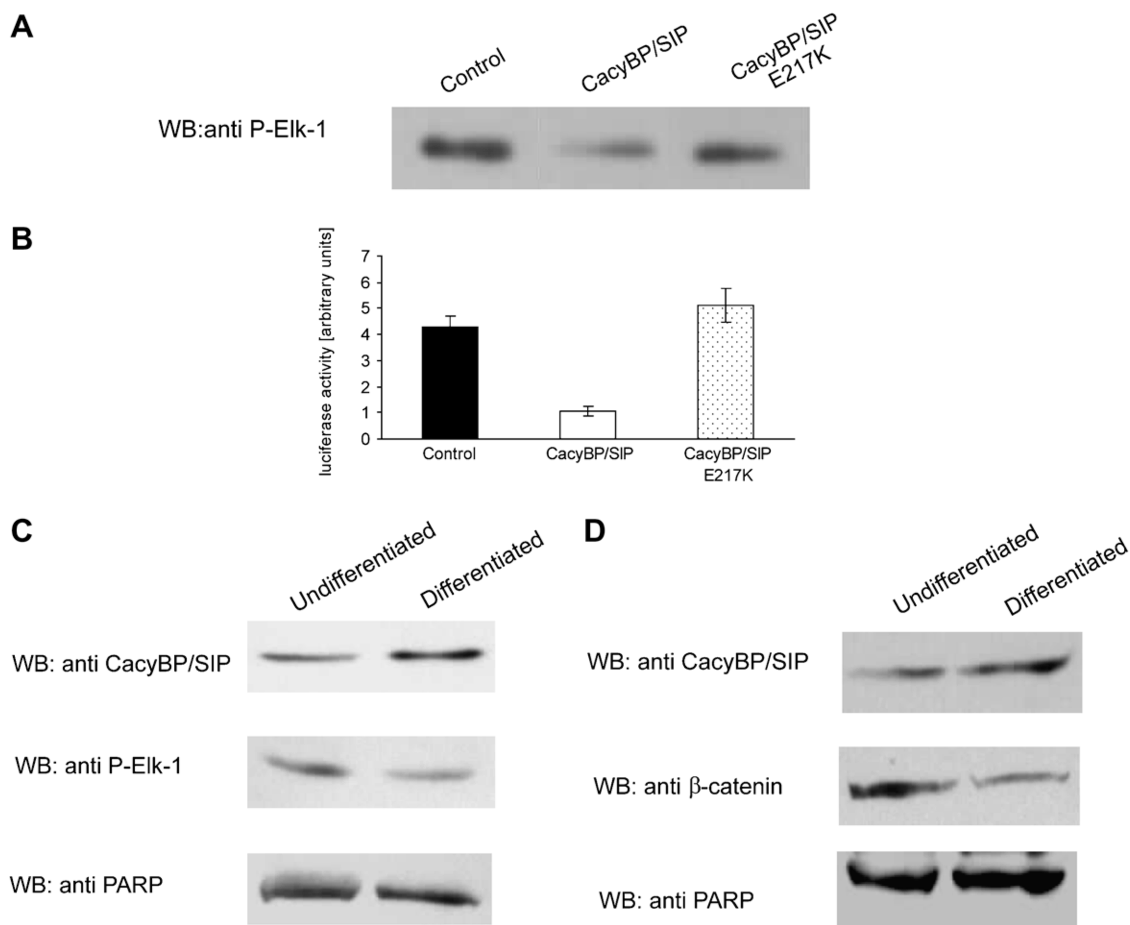

Fig. 4. (A) In vitro kinase assay showing the influence of CacyBP/SIP and its E217K mutant on the level of P-Elk-1. (B) Luciferase activity assay showing the effect of CacyBP/SIP and its E217K mutant on the transcriptional activity of Elk-1; black bar represents the activity in cells transfected with pcDNA3-HA-ERK2 alone (control), white bar shows the activity in cells co-transfected with pcDNA-CacyBP/SIP and dotted bar represents luciferase activity in cells co-transfected with pEGFP-CacyBP/SIP(E217K), results from three independent experiments are presented as a means ± SD. (C) Western blot showing the level of CacyBP/SIP and P-Elk-1 or (B) CacyBP/SIP and β-catenin in the nuclear fraction of undifferentiated and differentiated NB2a cells; 100 μg of protein was applied on the SDS-gel, staining with anti-PARP antibody shows that each lane contains a similar amount of nuclear proteins.

(Fig. 4B). In agreement with the results obtained from the in vitro assay, overexpression of the E217K mutant of CacyBP/SIP together with ERK2 did not have a significant effect on the luciferase signal/Elk-1 activity. CacyBP/SIP alone had no effect on this activity (data not shown).

To check the effect of CacyBP/SIP on the level of P-Elk-1 in the cell we used differentiated NB2a cells, in which both CacyBP/SIP and ERK1/2 are up-regulated. As it can be seen in Fig. 4C, the amount of CacyBP/SIP in the nuclear fraction inversely correlates with the level of P-Elk-1. The increase in the amount of CacyBP/SIP in the nuclear fraction of differentiated cells is about 40% and the decrease in P-Elk-1 level is about 30% as estimated by densitometric analysis of western blots from two independent experiments. Interestingly, in the nuclear fraction of differentiated cells the level of β-catenin is lower than in the same fraction of undifferentiated cells (Fig. 4D).

Discussion

In this work we showed that ERK1/2 bind to CacyBP/SIP and that a calcium binding protein, S100A6, competes for this binding. We also found that the E217K mutant of CacyBP/SIP does not bind significantly to ERK2 but it still binds to S100A6, indicating that the E217 residue is involved in the interaction with ERK2 but not in the interaction with S100A6. By performing molecular modeling we tried to explain the nature of the interaction between CacyBP/SIP and ERK1/2 and we found that it is based on electrostatic interactions, in contrast with the CacyBP/SIP-S100A6 binding which is supported by hydrophobic contacts [17]. Therefore, it is conceivable that S100A6 and ERK1/2 compete for the same domain of CacyBP/SIP but the E217K mutation disrupts only the binding with ERK1/2 leaving the hydrophobic interaction with S100A6 almost intact. Since S100A6 is a calcium binding protein, the results presented in this work suggest that changes in cellular calcium concentration might be important in the regulation of signaling pathway involving ERK1/2.

Data obtained from the in vitro kinase assay, luciferase experiment and cell fractionation clearly indicate that the interaction between CacyBP/SIP and ERK1/2 inhibits Elk-1 phosphorylation. Interestingly, the results of the in vitro kinase assay are in agreement with the data obtained for nuclear fraction of NB2a cells. The latter ones show an increased level of CacyBP/SIP and decreased level of P-Elk-1 in the nuclear fraction of differentiated cells in comparison to the nuclear fraction of undifferentiated ones. Thus, our results suggest that CacyBP/SIP might inhibit the Elk-1 phosphorylation/activity during the differentiation process in vivo.

Up to now, many reports have linked the activation of the ERK1/2–Elk-1 pathway with cell proliferation. For instance, it has been shown that phosphorylation/activation of Elk-1 by ERK1/2 is correlated with the activation of immediate early genes such as egr-1, c-fos, Mcl-1 [20,21]. There are also some reports showing that Elk-1 could be implicated in cell differentiation [22,23] but molecular mechanisms leading to this process are poorly understood. Our results show that CacyBP/SIP may attenuate the proliferative signals and direct cells to the differentiation pathway through its influence on Elk-1 activity. The concomitant decrease in β-catenin observed in the nuclear fraction of differentiated NB2a cells, an effect opposite to that seen in proliferating cells exhibiting a high level of this oncogen in the nucleus, would support this hypothesis. Since it was also shown, that CacyBP/SIP may be implicated in β-catenin degradation [2], the decreased level of β-catenin in the nuclear fraction might be a result of its higher degradation rate. To resolve this interesting issue, additional experiments should be performed. Altogether, our results suggest involvement of CacyBP/SIP and calcium binding proteins from the S100 family in the ERK1/2–Elk-1 pathway and provide a new insight into the molecular events leading to cell differentiation.

Acknowledgments

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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.bbrc.2009.01.026.

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