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ERK1/2 is dephosphorylated by a novel phosphatase – CacyBP/SIP

Ewa Kilanczyk [a], Slawomir Filipek [b], Anna Filipek [a,*]

[a] Nencki Institute of Experimental Biology, 3 Pasteur Street, 02-093 Warsaw, Poland
[b] Faculty of Chemistry, Warsaw University, 1 Pasteur Street, 02-093 Warsaw, Poland

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ABSTRACT

Recently, we have reported that the CacyBP/SIP protein binds ERK1/2 (Kilanczyk et al., BBRC, 2009). In this work we show that CacyBP/SIP exhibits a phosphatase activity toward ERK1/2 kinases while its E217K mutant does not. The K_m and V_{max} values established for a standard phosphatase substrate, *p*-NPP, are 16.9 ± 3.6 mM and 4.3 ± 0.4 μmol/min, respectively. The CacyBP/SIP phosphatase activity is decreased by okadaic acid (IC_{50} = 45 nM). Our experimental results are supported by a theoretical analysis which revealed important sequence similarities between CacyBP/SIP and the phosphatase-like proteins as well as certain MAP kinase phosphatases.

1. Introduction

The evolutionally highly conserved MAP kinase-dependent signaling pathways are involved in diverse cellular functions including gene expression, cell proliferation and differentiation. Specificity of these signaling pathways is achieved through binding interactions between individual MAP kinases and scaffolding proteins, substrates or phosphatases [1]. MAP kinase phosphatases (MKPs) play an important role in regulation of MAP kinase activity. Dephosphorylation of MAP kinases has been reported for serine/threonine phosphatases, tyrosine phosphatases or dual specificity threonine/tyrosine phosphatases [2]. Some MKPs are present and act predominantly in the cytoplasm, while some are nuclear. An example of a cytoplasmic phosphatase is MKP-3. The association of MKP-3 with ERK2 is highly specific and results in down-regulation of ERK2 kinase activity. MKP-1 and MKP-2 phosphatases are mainly present in the nuclear fraction. MKP-1 selectively associates with ERK1/2, JNK and p38, and inactivates these kinases in the nucleus [3]. The interaction of MKPs with MAP kinases is achieved through specific binding of positively charged amino acids (RRR motif/cluster) present in MKPs to an acidic domain present in a docking groove of MAP kinases [4,5].

CacyBP/SIP (Calcyclin binding protein/Siah-1 interacting protein) was discovered in Ehrlich ascities tumor cells [6] and then identified in other cells and tissues. CacyBP/SIP interacts with different ligands such as S100 proteins [7], Siah-1 [8], Skp1 [9], tubulin [10], ERK1/2 [11] and actin [12]. Binding of CacyBP/SIP with tubulin seems to be important in cytoskeleton rearrangement during differentiation of neuroblastoma NB2a cells [10]. This notion can be supported by the fact that increased level of CacyBP/SIP mRNA was found in some other cell lines during differentiation [13]. The interaction of CacyBP/SIP with ERK1/2 kinases and its influence on phosphorylation/transcriptional activity of Elk-1 might also suggest the involvement of CacyBP/SIP in cell differentiation [11].

Since CacyBP/SIP binds ERK1/2 and inhibits its activity, in this work we tried to establish whether CacyBP/SIP has phosphatase activity toward ERK1/2 kinases. At first we verified the phosphatase activity of CacyBP/SIP toward a standard substrate, *p*-NPP and calculated the K_m and V_{max} values as well as IC_{50} for the phosphatase inhibitor, okadaic acid. Then, we studied the influence of CacyBP/SIP and its E217K mutant on dephosphorylation of ^{32}P-ERK2.

2. Materials and methods

2.1. Cell-based experiments

Neuroblastoma NB2a cells were cultured and differentiated as described previously [10]. Transfection of NB2a cells was carried out using Lipofectamine2000 (Invitrogen) according to the manufacturer's protocol. For overexpression of CacyBP/SIP, undifferentiated NB2a cells were transfected with 10 μg of the plasmid and cultured for 24 h.

Abbreviations: ERK1/2, extracellular signal regulated kinases 1 and 2; MAPK, mitogen-activated protein kinase; MKP, MAPK phosphatase; MEK, MAPK/ERK kinases; PAGE, polyacrylamide gel electrophoresis; PARP, PolyADP-ribose polymerase; PBS, phosphate buffered saline; *p*-NPP, *p*-nitrophenyl phosphate; SDS, sodium dodecyl sulfate; TMB, tetramethylbenzidine.

* Corresponding author. Fax: +48 22 822 53 42.
E-mail address: a.filipek@nencki.gov.pl (A. Filipek).

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The nuclear fractions from NB2a cells were obtained using the NE-PER extraction reagents (Pierce) according to the 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 blots were quantified using the Ingenius Bio-Imaging (Syngene) and the Gene Tools software with PARP as a reference protein. The statistical data analysis was performed using Student's *t*-test. Mean values (±SEM) obtained from three independent experiments are presented as percentage of controls.

2.2. Plasmid construction and protein purification

Plasmid used for cell transfection, pcDNA-3.1-CacyBP/SIP was prepared as described previously [10]. Construction of plasmids for protein expression in bacteria and purification of recombinant proteins were also described earlier: CacyBP/SIP and its C-terminal fragment in [10], ERK2 and E217K mutant of CacyBP/SIP in [11].

2.3. SDS–PAGE and Western blotting

Gel electrophoresis with 10% (w/v) polyacrylamide containing 0.1% SDS was performed by the method of Laemmli [14]. Proteins were transferred electrophoretically onto nitrocellulose and identified using appropriate primary antibodies: rabbit anti-CacyBP/SIP affinity purified polyclonal antibody (1:100), 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), 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 a RETINA X-ray film.

2.4. In vitro dephosphorylation assay

Purified ERK2 kinase [11] was autophosphorylated for 30 min at 30 °C in 10 μl of a kinase buffer (Cell Signaling) supplemented with 0.2 mM ATP and 4 μCi ^{32}P[ATP] (specific radioactivity, 6000 Ci/mmol; Perkin Elmer). Then the sample was applied onto a Micro-Spin™ G-25 column (Amersham) and centrifuged at 3000*g* for 2 min to remove the excess of ATP. Next, 3 μg (5 μl) of either CacyBP/SIP or its fragment or Sgt1 (a homolog of CacyBP/SIP) [15], were added and incubation was carried out at 37 °C. After the indicated time the reaction was stopped by adding 10 μl of 4x concentrated sample buffer. When required, okadaic acid or NaF were added at a final concentration of 45 nM and 50 mM, respectively. Proteins were separated in SDS gel which was then dried and subjected to autoradiography for 14 h with an X-ray RETINA film at −70 °C.

2.5. p-NPP assay

The phosphatase activity of CacyBP/SIP was measured using para-nitrophenyl phosphate (*p*-NPP) (Sigma) as a substrate. The reaction was initiated by adding CacyBP/SIP (2 μg) to different concentrations (0.5, 1, 2, 5, 10, 15 and 20 mM) of *p*-NPP. The amount of the yellow end product, *p*-nitrophenol (*p*NP) released from *p*-NPP, was determined by measuring the absorbance at 405 nm after 3 min and quantified using the following relationship: OD 405 = E·b·[*p*NP] where $E = 17\,800\ M^{-1}\ cm^{-1}$, *b* = light path (cm), and [*p*NP] is the *p*NP concentration. To determine the kinetic parameters, K_m and V_{max}, the initial reaction velocities (v) were measured at various *p*-NPP concentrations.

To estimate the IC_{50} value, various concentrations of okadaic acid (2, 4, 8, 16, 32, 64, 128 nM) were added to the buffer containing 20 mM *p*-NPP and 2 μg of CacyBP/SIP, and phosphatase activity was measured as described above.

To check the phosphatase activity in the nuclear fractions, differentiated NB2a cells or NB2a cells overexpressing CacyBP/SIP were washed 3 times with PBS, and then the nuclear fractions were obtained as described above. Next, 100 μl (200 μg of protein) of the nuclear fractions were mixed with 500 μl of 10 mM *p*-NPP and absorbance at 405 nm was measured.

2.6. Staining of SDS gels with Pro-Q-Diamond

ERK2 kinase was autophosphorylated for 30 min at 30 °C in 10 μl of a kinase buffer (Cell Signaling) supplemented with 0.2 mM ATP. Then an increasing amount of CacyBP/SIP was added and dephosphorylation was carried out for 30 min at 37 °C. After that proteins were separated by SDS gel and stained first with Pro-Q-Diamond and then with SUPRO Ruby dye according to manufacturer's protocol. Shortly, the gel was fixed for 30 min with agitation at RT in 100 ml of solution containing 50% methanol and 10% acetic acid. Next, the gel was washed 3 times in water and stained overnight in 20 ml of an appropriate dye. After that the gel was destained by gentle agitation in the dark in 30 ml of solution containing 20% acetonitrile and 50 mM sodium acetate, pH 4.0 for 30 min at RT. After 3 washes in water the phosphorytated proteins were visualized using Phosphoimager, FLA-3000G, Fuji (Institute of Biochemistry and Biophysics, Warsaw). Results from three independent experiments were quantified using the Fla Tools software. The statistical data analysis was performed using the Student's test.

2.7. Theoretical analysis

All analyzed protein sequences were taken from the Swiss–Prot database [16]. The searches for similar sequences were performed using the BLAST network service at National Center for Biotechnology Information (NCBI) on non-redundant protein sequences. Two methods were used: protein–protein BLAST and Position-Specific Iterated BLAST (PSI-BLAST) [17]. Similarities between sequences in the final alignment were calculated using EMBOSS tools at the EBI network service.

3. Results

3.1. CacyBP/SIP has a phosphatase activity toward ERK1/2

Recently, it has been found that CacyBP/SIP binds ERK1/2 and inhibits phosphorylation of the Elk-1 transcription factor [11]. To examine the influence of CacyBP/SIP on the level of phosphorylated ERK1/2 (P-ERK1/2) we first checked the level of P-ERK1/2 in the nuclear fractions prepared from undifferentiated or differentiated NB2a cells. The latter cells exhibit a higher level of CacyBP/SIP [11]. As it can be seen in Fig. 1A, a lower level of P-ERK1/2 is detected in differentiated NB2a cells than in undifferentiated ones. Densitometric analysis of the results obtained from three independent experiments revealed that the intensity of the P-ERK1/2 band in differentiated cells is only 58% (±14%) of the intensity of the band seen in undifferentiated cells. To check if the phosphatase activity in the nuclear fractions of differentiated NB2a cells and of NB2a cells overexpressing CacyBP/SIP is higher than in the nuclear fraction of undifferentiated ones we applied the *p*-NPP assay. As it can be seen in Fig. 1B the phosphatase

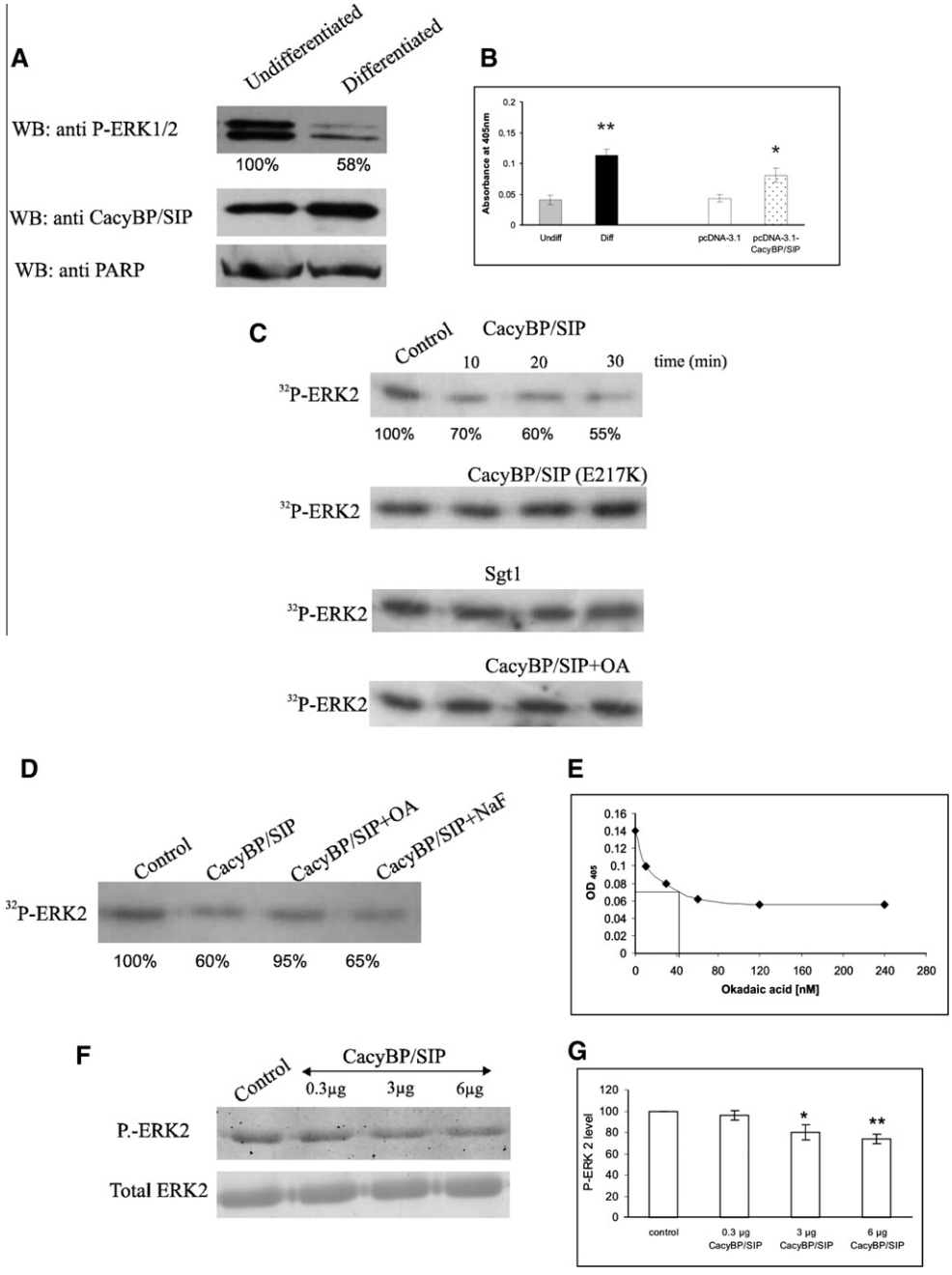

Fig. 1. (A) Western blot showing the level of CacyBP/SIP and P-ERK1/2 in the nuclear fractions of NB2a cells. Staining with anti-PARP antibody shows that each lane contains a similar amount of nuclear proteins. Mean P-ERK1/2 band intensities obtained from three independent experiments (presented as percentage of mean control value) are shown. (B) phosphatase activity in nuclear fractions prepared from undifferentiated (grey bar), differentiated NB2a cells (black bar) and from undifferentiated NB2a cells transfected with control plasmid (white bar) or with pcDNA-3.1-CacyBP/SIP (dotted bar) assessed by *p*-NPP assay. Diff – differentiated cells, Undiff - undifferentiated cells. Results of three independent experiments are presented as a mean ± SEM; $^{**}p \leqslant 0.01$, $^{*}p \leqslant 0.05$. (C) The level of ^{32}P-ERK2 after 10, 20 or 30 min incubation with CacyBP/SIP, its E217K mutant, Sgt1 or CacyBP/SIP and okadaic acid (OA) estimated by autoradiography. (D) The level of ^{32}P-ERK2 after 30 min incubation with CacyBP/SIP, CacyBP/SIP and okadaic acid (OA) or CacyBP/SIP and NaF, estimated by autoradiography. For C and D, mean ^{32}P-ERK2 band intensities (presented as percentage of mean control value) obtained from three independent experiments are shown. (E) The effect of different concentrations of okadaic acid (OA) on CacyBP/SIP phosphatase activity estimated by *p*-NPP assay. (F) The level of P-ERK2 estimated after incubation with indicated amounts of CacyBP/SIP and staining of SDS-gel with Pro-Q-Diamond and SUPRO Ruby dye. The staining with SUPRO Ruby dye shows that the amount of total ERK2 in each lane is similar. (G) Statistical analysis of the Pro-Q-Diamond signal intensities (relative to control taken as 100%) estimated by densitometry. Results of three independent experiments are presented as a mean ± SEM; $^{**}p \leqslant 0.01$, $^{*}p \leqslant 0.05$.

activity is higher in differentiated cells and in cells overexpressing CacyBP/SIP.

In the next step we checked whether purified CacyBP/SIP exhibits its phosphatase activity toward the *p*-NPP substrate. We found that indeed it is able to hydrolyze *p*-NPP which allowed us to calculate the K_m and V_{max} values to be 16.9 ± 3.6 mM and 4.3 ± 0.4 µmol/min, respectively. To establish the effect of CacyBP/SIP on ERK2 dephosphorylation, the ERK2 kinase was autophosphorylated for 30 min in the presence of ^{32}P[ATP] and then CacyBP/SIP or its E217K mutant (which is defective in ERK1/2 binding [11]) or Sgt1 (the CacyBP/SIP homolog) were added. Fig. 1C shows that in the presence of CacyBP/SIP the level of ^{32}P-ERK2 dropped in time to 70% (±5%)–55% (±6%) of the control value. When CacyBP/SIP was replaced by the E217K CacyBP/SIP mutant or Sgt1, the level

of ^{32}P-ERK2 was similar as in the control (Fig. 1C). Moreover, CacyBP/SIP, similarly to other phosphatases, seems to be sensitive to the phosphatase inhibitor, okadaic acid, since the level of ^{32}P-ERK2 did not change when this reagent was included in the assay (Fig. 1C). When NaF was included in the assay the phosphatase activity of CacyBP/SIP was unchanged suggesting that NaF does not act as CacyBP/SIP inhibitor (Fig. 1D). The IC_{50} calculated for okadaic acid was 45 nM (Fig. 1E). The effect of CacyBP/SIP on P-ERK2 level was also confirmed by the Pro-Q-Diamond staining of phosphorylated proteins (Fig. 1F) and appeared to be dose dependent (Fig. 1G). Thus, the results obtained in *in vitro* experiments using ^{32}P[ATP] are in agreement with data showing a lower level of P-ERK1/2 in the nuclear fraction of NB2a cells containing higher level of CacyBP/SIP.

3.2. Comparison of the amino acid sequence of CacyBP/SIP C-terminal fragment with other phosphatases

Data showing that the E217K mutant of CacyBP/SIP did not exhibit the phosphatase activity suggest that the C-terminal fragment of this protein plays an important role in ERK1/2 dephosphorylation. Thus, we searched for possible sequence similarities between this fragment and various phosphatases. Such analysis revealed a high number of identical residues (about 50%) within a 20 amino acid long sequence present in the C-terminal fragments of human and mouse CacyBP/SIP and three phosphatase-like proteins (Fig. 2A). Both CacyBP/SIP sequences harbor a characteristic, positively charged KR motif in keeping with the (K/R)R motif present in the C-termini of the phosphatase-like proteins and in the MKP-1 and MKP-2 phosphatases (Fig. 2A). The cluster of positively charged residues present in the C-terminal fragment is accompanied by a glutamic acid residue (E) present at the same distance from the positively charged amino acids in almost all sequences shown in this figure. This glutamic acid (E) residue in mouse CacyBP/SIP is located at position 217 (in the C-terminal part of the molecule) and mutation of this residue abolishes the phosphatase activity of CacyBP/IP. In agreement with this theoretical analysis are the experimental results showing that the C-terminal fragment (residues 178–229) of CacyBP/SIP binds ERK2 (Fig. 2B) and that it exhibits phosphatase activity (Fig. 2C).

4. Discussion

The magnitude and duration of ERK1/2 kinase activity is determined by the balance between the activity of MEK1/2, the kinases that phosphorylate and activate ERK1/2, and the activity of phosphatases that can dephosphorylate and inactivate ERK1/2. In this work we show for the first time that CacyBP/SIP exhibits phosphatase activity toward ERK1/2 kinases. Theoretical analysis showed that CacyBP/SIP shares high sequence similarity with some phosphatase-like proteins within its C-terminal fragment, which contains a characteristic (K/R)R motif. The motif of two positively charged residues may indicate phosphatase activity similar to the activity of more typical phosphatases [3]. Sequence analysis

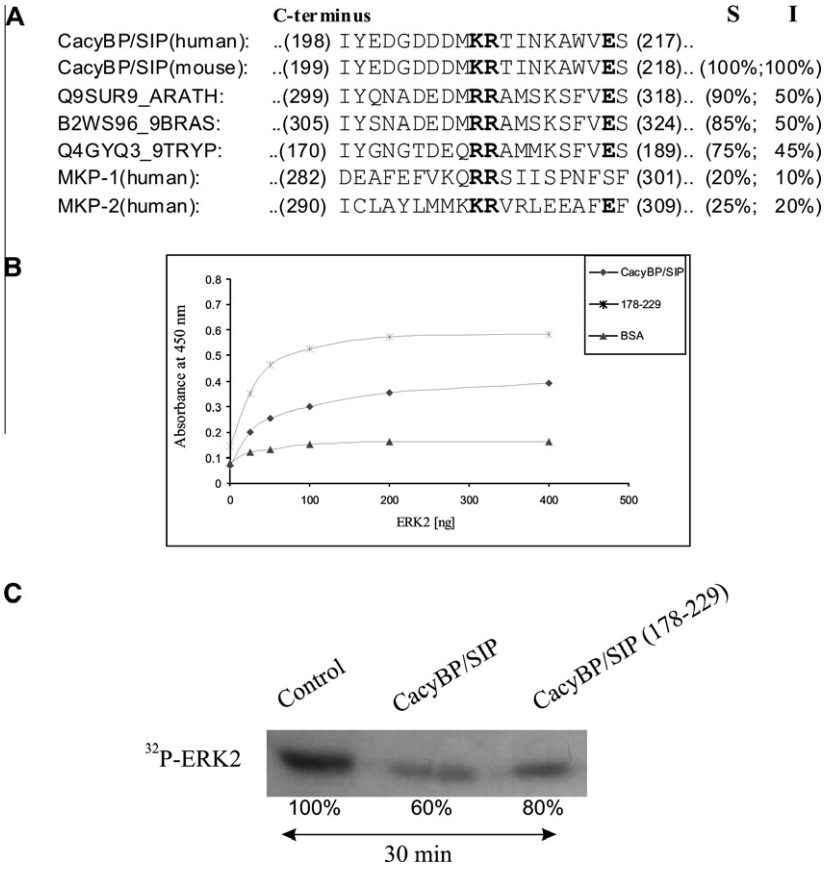

Fig. 2. (A) Comparison of a 20 amino acid long sequence from the C-terminal fragment of CacyBP/SIP with sequences of three phosphatase-like proteins (named by symbols from Swiss–Prot database) and of MKP-1 and MKP-2. The residues forming the motifs found in phosphatases are bolded. Numbers at the end of sequences describe similarity (S) and identity (I) to human CacyBP/SIP sequence. (B) Interaction of the full length CacyBP/SIP and its C-terminal fragment with ERK2 estimated by ELISA assay (performed as described in [11]). (C) The level of ^{32}P-ERK2 after incubation with CacyBP/SIP or its C-terminal fragment (178–229) estimated by autoradiography. Mean ^{32}P-ERK2 band intensities (presented as percentage of mean control value) obtained from three independent experiments are shown. In B and C a representative experiment out of three performed is shown.

revealed that the (K/R)R motif is accompanied by a glutamic acid residue (E) located at a constant distance from the positively charged residues We speculate that the positively charged residues may pull the phosphate group whereas the side chain of glutamic acid may bind to the positively charged carbon atom of a phospho-amino-acid to perform cleavage. Interestingly, this characteristic motif is not present in the amino acid sequence of the Sgt1 protein (data not shown), a homolog of CacyBP/SIP without phosphatase activity. Thus, our theoretical analysis confirms that CacyBP/SIP is a novel phosphatase for ERK1/2 kinases and could modulate their function in different cellular processes. In this work we found that both the CacyBP/SIP protein and the C-terminal fragment of CacyBP/SIP (178–229) exhibit phosphatase activity. Our results show that the K_m and V_{max} values for CacyBP/SIP estimated in an *in vitro* assay with *p*-NPP are 16.9 ± 3.6 mM and 4.3 ± 0.4 μmol/min, respectively. Similar K_m and V_{max} values were obtained for the AcP phosphatase [18]. Our results show also that okadaic acid, but not NaF, inhibits the enzymatic activity of CacyBP/SIP. The IC_{50} value estimated for okadaic acid in the *p*-NPP assay is about 45 nM and it is in the range found for other phosphatases [19]. Interestingly, we found that the tryptophan fluorescence intensity of CacyBP/SIP and of its C-terminal fragment decreases after addition of okadaic acid by about 15% and 10%, respectively (data not shown). In the case of the E217K CacyBP/SIP mutant the fluorescence intensity decreases only by 4–5% (data not shown). This indicates that okadaic acid indeed binds to CacyBP/SIP and to its C-terminal fragment and induces conformational changes which in consequence cause changes in fluorescence intensity.

Based on our earlier [11] and present results we postulate that dephosphorylation of ERK1/2 by CacyBP/SIP in the nuclear fraction of NB2a cells decreases the activity of Elk-1 and the level of β-catenin, two pro-proliferating agents. This in consequence might inhibit cell division and drive NB2a cells into differentiation. Interestingly, in PC12 cells, increased expression of MKP-1 inhibited DNA synthesis and cell division, and could contribute to differentiation of these cells [20]. Altogether, our results show that CacyBP/SIP is a novel phosphatase for ERK1/2 kinases. It seems also that, as a phosphatase, CacyBP/SIP might play a role as a new negative modulator of ERK1/2 kinases and that, in consequence, this protein might be implicated in differentiation of neuroblastoma NB2a cells.

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