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Translocation and Phosphorylation of Calcyclin Binding Protein during Retinoic Acid-induced Neuronal Differentiation of Neuroblastoma SH-SY5Y Cells

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For better understanding of functions of the Calcyclin Binding Protein (CacyBP) and exploring its possible roles in neuronal differentiation, the subcellular localization of human CacyBP was examined in retinoic acid(RA)-induced and uninduced neuroblastoma SH-SY5Y cells. Immunostaining indicated that CacyBP was present in the cytoplasm of uninduced SH-SY5Y cells, in which the resting Ca^{2+} concentration was relatively lower than that of RA-induced cells. After the RA induction, immunostaining was seen in both the nucleus and cytoplasm. In the RA-induced differentiated SH-SY5Y cells, CacyBP was phosphorylated on serine residue(s), while it existed in a dephosphorylated form in normal (uninduced) cells. Thus, the phosphorylation of CacyBP occurs when it is translocated to the nuclear region. The translocation of CacyBP during the RA-induced differentiation of SH-SY5Y cells suggested that this protein might play a role in neuronal differentiation.

Keywords: Human CacyBP, Neuroblastoma SH-SY5Y, Phosphorylation, RA-induced differentiation, Translocation

Introduction

Calcyclin (S100A6) is a calcium-binding protein of the EF-hand type that belongs to the S100 family (Kuznicki, 1996; Donato, 2001). It has been shown that S100 proteins are associated with cell cycle progression, differentiation, metabolism (Zimmer *et al*., 1995; Schafer *et al*., 1996; Donato, 2001), and the induction of metastatic phenotype (Gibbs *et al*., 1994; Ilg *et al*., 1996). In contrast to calmodulin, S100 proteins exhibit cell and tissue specific expressions.

The Calcyclin Binding Protein (CacyBP) was originally discovered in the cytosolic fraction of Ehrlich ascites tumor cells, mouse brain, and spleen (Filipek *et al*., 1996). The cDNA clone of CacyBP was isolated from the mouse brain library and sequenced (Filipek *et al*., 1998). The recombinant CacyBP interacted with calcyclin *in vitro* at the micromolar Ca^{2+} concentration, indicating that this interaction may indeed occur physiologically. We first cloned the human homolog of mouse CacyBP during differentiation of the gliomablastoma BT325 cell (obtained from Beijing Tiantan Hospital) by DDRT-PCR. Almost at the same time, a protein that was called SIP (Siah-1 Interacting Protein), a component of the β-catenin ubiquitin degradation pathway, was reported (Matsuzawa *et al*., 2001). It was, in fact, the human CacyBP. It showed a 93% sequence identity with mouse CacyBP. Two independent reports showed that the level of CacyBP increased upon erythropoietin receptor activation (Xia *et al*., 2000; Pircher *et al*., 2001). Recently, the subcellular localization of CacyBP was examined in neurons and neuroblastoma NB-2a cells at different $[Ca^{2+}]_i$ (Filipek *et al*., 2002). Immunostaining indicated that CacyBP was present in the cytoplasm of unstimulated cultured neurons or neuroblastoma NB-2a cells, in which resting $[Ca^{2+}]_i$ was relatively low. When $[Ca^{2+}]_i$ increased to above 300 nM, then the immunostaining was mainly seen as a ring around the nucleus. The phosphorylation of CacyBP occurred in the same range of $[Ca^{2+}]_i$ that leads to its perinuclear translocation. Since the Ca^{2+}-dependent cellular differentiation was previously described (Mizuno *et al*., 1989), then the interaction of CacyBP with an EF-hand calcium binding protein raises the possibility that CacyBP may function *via* Ca^{2+}-dependent interactions in cellular differentiation. Therefore, in this work, we studied the intracellular localization of CacyBP during the retinoic acid (RA)-induced differentiation of human neuroblastoma cells SH-SY5Y. Using endogenous expression and immunostaining, we show

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that the cellular localization of CacyBP is changed during differentiation. We also found that the phosphorylation of this protein on serine residue(s) occurred with its nuclear translocation during retinoic acid (RA)-induced neuronal differentiation.

Materials and Methods

Cell culture and RA induction Undifferentiated human neuroblastoma cells, SH-SY5Y, were maintained in DMEM that was supplemented with 10% fetal bovine serum, penicillin (100 μg/ml), and streptomycin (100 μg/ml). All of the cultures were maintained in the presence of 5% CO_2 at 37°C. The SH-SY5Y cells were induced for differentiation by all-trans retinoic acid (RA) treatment, as previously described in detail (Gao *et al*., 1998).

Cellular extract preparation and Western blot To prepare the protein extracts for a Western blot, the cells were harvested and washed with PBS. Next, the cells were lysed in a buffer containing 20 mM Tris-HCl pH 7.5, 8 mM $MgCl_2$, 150 mM NaCl, 0.2 mM EGTA, 1% Nonidet P-40, and protease inhibitors [leupeptin (10 mg/l), aprotinin (5 mg/l), soybean trypsin inhibitor (20 mg/l), and phenylmethylsulfonyl fluoride (1 mM)]. The extracts were obtained by centrifuging at 12,000 rpm for 25 min at 4°C. The extracts were loaded onto SDS-polyacrylamide gels, and the electrophoresed proteins were electrotransferred to nitrocellulose membranes (Hybond ECL, Amersham Biosciences, Buckinghamshire, UK). The membrane was blocked with PBS, 0.1% Tween 20, and 5% non-fat dry milk for at least 1 h. The membrane was incubated with the primary antibody in the previously mentioned solution at 4°C for 16 h. After washing with PBS containing 0.1% Tween 20, the filter was incubated with either an alkaline phosphatase-conjugated or a horseradish peroxidase-conjugated secondary antibody in PBS, 0.1% Tween 20, and 5% non-fat dry milk for 1 h at room temperature. The signal development was performed as recommended by the manufacturer.

Immunocytochemistry The SH-SY5Y cells were plated onto poly-L-lysine-coated coverslips. The cells were fixed with 4% paraformaldehyde in PBS (pH 7.4) for 20 min at room temperature. The coverslips were washed with PBS and permeabilized for 10 min with 0.5% Triton X-100 in PBS. The cells were washed twice with PBS, incubated with 3% bovine serum albumin in PBS for 1 h, and then incubated with CacyBP antibodies (1 : 200). After washing (three times for 10 min in PBS), the cells were incubated with FITC-conjugated anti-rabbit antibodies (1 : 200) (Santa Cruz Biotech., Santa Cruz, USA) and mounted on glass slides with a mixture of glycerol and polyvinyl alcohol containing DABCO (1,4-diazobicylo-[2,2,2,]-octane). For the control experiments, the cells were incubated with pre-immune serum. The cells were analyzed under a Leica TCS NT confocal laser microscope.

Cytosolic free Ca^{2+} measurement The SH-SY5Y cells (untreated and treated with RA) were loaded with fura-3 (Molecular Probes, Eugene, USA) during an 1-h incubation at 37°C in PBS. The coverslip with the loaded cells was then placed on the homoeothermic platform of a confocal laser microscope. Fura-3 was successively excited at 488 nm by means of two narrow band-pass filters. The emitted fluorescence was filtered through a 520 nm filter and captured at a resolution of 512×480 pixels, digitized into 256 gray levels and analyzed with software. The Ca^{2+} concentration was presented by averaging the fluorescence intensity of all of the pixels in the undifferentiated or differentiated SH-SY5Y cells.

Immunoprecipitation and protein phosphorylation The uninduced and induced cells were washed in PBS, then harvested and lysed on ice in a buffer containing 20 mM Tris-HCl pH7.5, 8 mM $MgCl_2$, 150 mM NaCl, 0.2 mM EGTA, and 1% Nonidet P-40. The extracts were centrifuged at 12,000 rpm for 25 min at 4°C, in a micro centrifuge. The supernatants were used for an immunoprecipitation assay after adding the protease inhibitors (10 mg/l leupeptin, 5 mg/l aprotinin, 20 mg/l soybean trypsin inhibitor, 1 mM phenylmethylsulfonyl fluoride), and phosphatase inhibitors (0.3 mM okadaic acid, 200 M Na_3VO_4, 5 mM NaF). First, the solutions were incubated with protein A-Sepharose for 1 h at 4°C (pre-clearance). The unbound fractions were incubated with serum containing antibodies against CacyBP for 1.5 h at 4°C, and then for 1 h at 4°C with a new portion of protein A-Sepharose. The resin was washed three times in a buffer containing 20 mM Tris-HCl pH 7.5 and 150 mM NaCl, twice in a buffer containing 20 mM Tris-HCl pH 7.5 and 500 mM NaCl, and finally in 20 mM Tris-HCl at pH 7.5. All of the buffers that were used were supplemented with protease and phosphatase inhibitors. The resin containing the bound proteins was solubilized in an SDS sample buffer, boiled for 5 min at 98°C, and applied on the SDS polyacrylamide gel (Lee *et al*., 2001). The phosphorylation of CacyBP was analyzed using Western blot technique with monoclonal antibodies against phosphorylated serine residue (Calbiochem, San Diego, USA), as described in the original protocols.

Results

Subcellular localization of CacyBP during RA-induced differentiation of neuroblastoma SH-SY5Y cells To learn more about the relationship between CacyBP and cellular differentiation (particularly neuronal differentiation), we first studied the intracellular localization of this protein during the retinoic acid-induced differentiation of neuroblastoma SH-SY5Y cells. CacyBP is endogenously expressed in neuroblastoma SH-SY5Y cells, observed by Western blotting in the extracts of these cells (Fig. 1).

The neuroblastoma SH-SY5Y cells were induced with all-trans retinoic acid for 7 days, fixed, and then stained with antibodies against CacyBP. In the uninduced cells, immunostaining was mainly observed in the cytoplasm (Fig. 2a). In the RA-induced cells, on the other hand, the endogenous CacyBP was detected in both the nuclear and cytoplasmic regions (Fig. 2b), which indicates the translocation of this protein toward the nucleus upon differentiation.

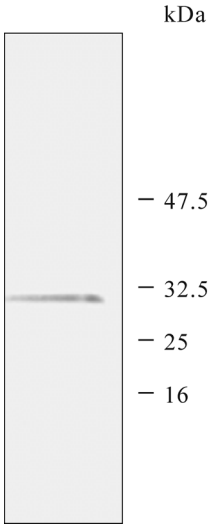

Fig. 1. CacyBP endogenously expressed in neuroblastoma SH-SY5Y cells, detected by Western blot using anti-CacyBP polyclonal antibodies.

Ca^{2+} concentration increases during differentiation The intracellular Ca^{2+} concentration (presented by average fluorescence intensity) increased by $39 \pm 2\%$ in the RA-induced SH-SY5Y cells when compared to the uninduced cells (data not shown).

Phosphorylation of CacyBP in neuroblastoma SH-SY5Y cells One possible mechanism for the Ca^{2+}-dependent localization of CacyBP in the nuclear region is protein phosphorylated. To examine if CacyBP might exist in a phosphorylation form *in vivo*, the extracts of the uninduced and RA-induced SH-SY5Y cells were immunoprecipitated with antibodies against CacyBP; the precipitated proteins were analyzed by anti-phosphoserine antibodies. In the case of the RA-induced SH-SY5Y cells, CacyBP phosphoserine immunoreactivity was observed. The result indicates that CacyBP is phosphorylated on serine residue(s) in RA-induced SH-SY5Y cells. In the normal (uninduced) cells, the immunoprecipitated CacyBP contained no phosphoserine immunoreactivity (Fig. 3b). Thus, we find that CacyBP phosphorylation occurs when it is translocated to the nuclear region during the RA-induced differentiation of neuroblastoma SH-SY5Y cells

Discussion

Cell differentiation is a complex process that is regulated by an interplay among intrinsic cellular programs, cell-cell and cell-substrate interactions, and a plethora of soluble extracellular signaling molecules including hormones, growth factors, cytokines, trophic factors, and morphogens. Retinoic acid (RA), a metabolic derivative of vitamin A, plays a crucial role in the development and differentiation of the nervous

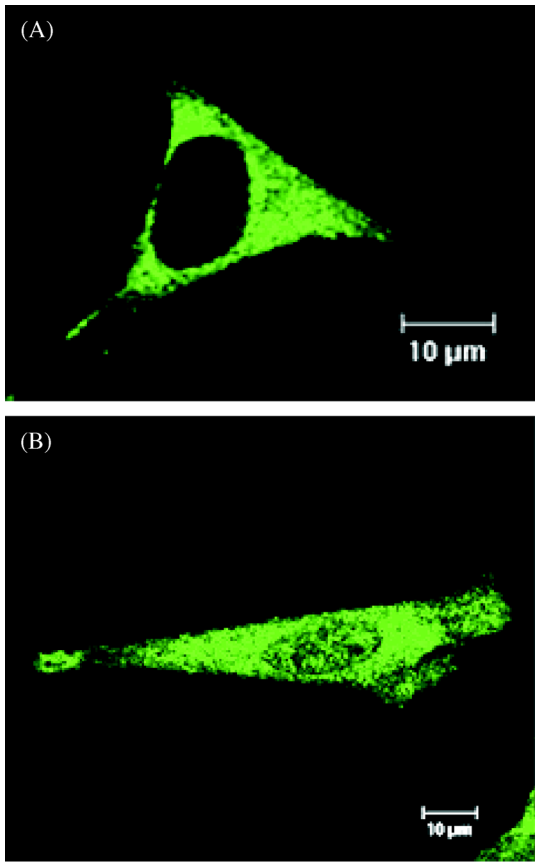

Fig. 2. Subcellular localization of CacyBP in neuroblastoma SH-SY5Y cells. Endogenous protein was identified by immunocytochemistry. (A) Uninduced SH-SY5Y cell; (B) SH-SY5Y cell was induced by all-trans retinoic acid for 7 days.

system. *In vitro*, RA also plays a prominent role in regulating the transition from the proliferating precursor cell to the postmitotic differentiated cell. There are many examples in literature of the distinct cell types whose differentiation is under the control of RA. SH-SY5Y cells, a neuroblastic subclone of the neuroblastoma cell line SK-N-SH, withdraw from the cell cycle and exhibit a distinct neuronal phenotype when treated with different agents, such as neuortrophic factors (Kaplan *et al.*, 1993), retinoic acid (Kaplan *et al.*, 1993), phorbol ester (Pahlman *et al.*, 1981), or staurosporine (Jalava *et al.*, 1992; Jalava *et al.*, 1993). We first cloned the human CacyBP gene during the differentiation of the gliomablastoma cell, BT325. Calcycline (S100A6), one of the interacting proteins of CacyBP, is associated with cell differentiation, and a recent study discovered that CacyBP might be involved in erythroid cell differentiation. This progress on CacyBP encourages us to explore its possible role in neuronal differentiation and their relationships.

In normal SH-SY5Y cells, CacyBP was distributed throughout the cytoplasm, but after induction with all-trans RA, the immunoreactivity was visible in both the nucleus and cytoplasm. The intracellular Ca^{2+} level in the uninduced cells

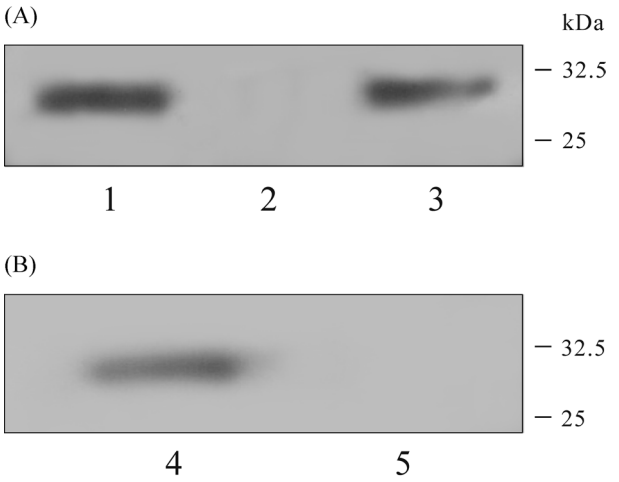

Fig. 3. Study of CacyBP phosphorylation in SH-SY5Y cells. Endogenous CacyBP was immunoprecipitated from the extract of uninduced SH-SY5Y cells (lanes 3, 5) or RA-induced cells (lanes 1, 4) using anti-CacyBP antibodies. Immunoprecipitated proteins were separated on 12% SDS-PAGE, blotted onto nitrocellulose filter, and probed with antibodies against CacyBP (A) or phosphoserine (B). Lane 2: prestained protein marker.

was relatively low. Upon RA induction, it rose to a higher level. The results are somewhat consistent with Filipek's study; CacyBP is present throughout the cytoplasm at low $[Ca^{2+}]_i$, and is translocated into the perinuclear region when $[Ca^{2+}]_i$ is increased (Filipek *et al*., 2002). What mechanism might be responsible for the translocation of CacyBP? There is a possibility that some post-translational modifications of CacyBP might regulate its localization within the cell. According to a recent study, CacyBP distribution within the cell is modulated by the changes in $[Ca^{2+}]_i$ This phenomenon might be stimulated or regulated by the phosphorylation process (Filipek *et al*., 2002). Our study found that CacyBP is phosphorylated on serine residue(s) in RA-induced neuroblastoma SH-SY5Y cells, and exists in a dephosphorylated form in normal cells. In other words, reversible serine phosphorylation occurs when a portion of CacyBP translocates to the nuclear region. An analysis of the CacyBP sequence identified a nuclear localization signal (NLS) and potential phosphorylation sites for protein kinase C. However, the relationship among the putative NLS-mediated localization, Ca^{2+}-dependent translocation of CacyBP, and protein phosphorylation remains an unresolved issue, as does the physiological significance of these observations. For example, the treatment of PC12 cells with RA induced a sustained increase of $[Ca^{2+}]$ over several days and Ca^{2+}-dependent phosphorylation of a protein with an apparent molecular weight of 27 kDa (Mizuno *et al*., 1989). In this case, RA-induced neuronal differentiation has some relationship with $[Ca^{2+}]$ and protein phosphorylation. In this paper, changes in the state of the CacyBP phosphorylation and subcellular localization during RA-induced neuronal differentiation suggest that this protein might be involved in the differentiation of neuronal cells. Future work will be focused on the physiological significance of these observations.

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