Acta Biochim Biophys Sin (2009): 883–891 | © The Author 2009. Published by ABBS Editorial Office in association with Oxford University Press on behalf of the Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. DOI: 10.1093/abbs/gmp082.

RanBPM is an acetylcholinesterase-interacting protein that translocates into the nucleus during apoptosis

Xiaowen Gong†, Weiyuan Ye†, Haibo Zhou, Xiaohui Ren, Zhigang Li, Weiyin Zhou, Jun Wu, Yicheng Gong, Qi Ouyang, Xiaolin Zhao, and Xuejun Zhang*

Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 200031, China

†These authors contributed equally to this work.

*Correspondence address. Tel: +86-21-54921403; Fax: +86-21-54921403; E-mail: xjzhang@sibs.ac.cn

Acetylcholinesterase (AChE) expression may be induced during apoptosis in various cell types. Here, we used the C-terminal of AChE to screen the human fetal brain library and found that it interacted with Ran-binding protein in the microtubule-organizing center (RanBPM). This interaction was further confirmed by coimmunoprecipitation analysis. In HEK293T cells, RanBPM and AChE were heterogeneously expressed in the cisplatin-untreated cytoplasmic extracts and in the cisplatin-treated cytoplasmic or nuclear extracts. Our previous studies performed using morphologic methods have shown that AChE translocates from the cytoplasm to the nucleus during apoptosis. Taken together, these results suggest that RanBPM is an AChE-interacting protein that is translocated from the cytoplasm into the nucleus during apoptosis, similar to the translocation observed in case of AChE.

Keywords AChE; RanBPM; yeast two-hybrid; cisplatin; apoptosis

Received: April 21, 2009 Accepted: June 21, 2009

Introduction

Acetylcholinesterase (AChE, EC 3.1.1.7) is a carboxylesterase that plays an essential role in acetylcholine-mediated neurotransmission. *AChE* transcripts are classified into R, H, and T transcripts, which are formed by alternative splicing of a single gene in vertebrates [1]. AChE-T is expressed in muscles and the nervous system; therefore, it represents the functional cholinesterase species in the cholinergic system [2,3]. AChE-T is also referred to as AChE-S (synaptic) [4].

Besides the function of hydrolyzing acetylcholine, AChE has been reported to perform other non-catalytic roles [5], such as promoting cell adhesion and neurite outgrowth [6], amyloidosis [7], and apoptosis [8].

In our previous study, the induction of apoptosis by various stimuli led to the expression of AChE-S in various cells [8]. After the cell has committed to apoptosis, AChE first appears in the cytoplasm followed by its appearance in the nucleus or the apoptotic bodies [8,9]. The blockage of AChE expression by antisense or the inhibition of AChE activity by pharmacological inhibitors can prevent the induction of apoptosis [8], suggesting that AChE expression is not just a consequence of apoptosis. Moreover, the silencing of the *AChE* gene by siRNA indicated that AChE may play a critical role in the apoptosome [10]. However, the function of AChE has not been elucidated.

Interactions of AChE with a number of proteins and peptides have been reported, including laminin [11–13], collagen IV [11], the nicotinic acetylcholine receptor [14], the RACK1 [15], the C-terminal binding protein (CtBP) [16], and the amyloid β-peptide (Aβ) [17]. Laminin or collagen IV was found to bind AChE on or near the site of the peripheral anionic site (PAS) of AChE [11–13]. PAS is a specific motif, located close to the rim of the active-site gorge of the enzyme. Laminin and collagen IV are the most abundant components of the basement membrane (BM) and it is well documented that BM components promote cell migration and neurite outgrowth [18], so the interaction between AChE and laminin or collagen IV has the possible roles in neurite outgrowth and synaptogenesis. An AChE-peptide

derived from the C-terminus of AChE, a novel nicotinic acetylcholine receptor ligand, is bioactive in a ligand-specific and concentration-dependent manner [14]. The nanomolar pathophysiological concentrations of AChE-peptide induce neuronal damage via a predominantly apoptotic mechanism involving α7 nicotinic AChR (nAChR) activation [19,20]. The scaffold protein the receptor for activated C kinase 1 (RACK1) interacts with the C-terminal domain unique to AChE-R (different from the C-terminal domain of AChE-S) [15,21,22]. AChE-R correlates with intensified fear-induced conflict behavior through AChE-R/RACK1/PKC βII complexes [15] and has a genotoxic resistance by competing with endogenous RACK1 for p73 interaction [22]. The binding of CtBP to the C-terminal of AChE-S can modify Ikaros functions, thereby causing T lymphopenia [16]. Aβ interacts with AChE on the site of Ω-loop located in the vicinity of the PAS of AChE, which promotes Aβ fibril formation [17].

Butyrylcholinesterase (BChE; EC 3.1.1.8) is homologous to AChE, and compensates, to some degree, for the lack of AChE in synapses and neuromuscular junctions. BChE lacks the peripheral site (PAS) which has been shown to be significant in the non-synaptic functions of AChE [23]. Although BChE is closely homologous to AChE (70% identity), it does not promote cell adhesion [24,25]. It does not bind laminin *in vitro* [11], nor does this protein dock with Gas6, the ligand for Mer. BChE-peptide, at lower concentrations, had little physiological effects on endogenously expressed α7 nAChR [14]. The BChE gene is amplified or abnormally expressed in tumorigenesis and some neuronal disorders, and the suppression of BChE increases the rate of cell death [25]. However, the blockage of AChE expression can prevent the induction of apoptosis [8]. So BChE is unlikely to replace AChE in neurite outgrowth, amyloidosis, and apoptosis.

To study the mechanisms of AChE during apoptosis, we used a yeast two-hybrid system to identify the proteins that interact with AChE. Ran-binding protein in the microtubule-organizing center (RanBPM) was originally identified in a yeast two hybrid searching for novel binding partners of Ran GTPase [26]. RanBPM is a multi-domain intracellular protein that has been found to act as a scaffold for certain tyrosine kinases [27,28], adhesion receptors [29,30], and nuclear-signaling proteins [31,32]. However, unlike most other identified Ran-binding proteins, RanBPM is not involved in nuclear-cytoplasmic transport [33,34].

In this article, we established that RanBPM is an AChE-interacting protein that is translocated from the cytoplasm into the nucleus during apoptosis, similar to the translocation of AChE.

Materials and Methods

Plasmid construction

pACT2-RanBPM was isolated from a human fetal brain matchmaker cDNA library (Clontech, Mountain View, USA) by yeast two-hybrid. HA-RanBPM or Myc-RanBPM was constructed by transferring RanBPM insert from pACT2-RanBPM into a pCMV-HA (Clontech) or pCMV-Myc vector (Clontech) by *Sfi*I and *Xho*I digestion. In addition, pcDNA3-Myc-AChE$_{C\text{-terminal}}$ was constructed by transferring the C-terminus of human AChE to pcDNA3-Myc (derived from pcDNA3.0, Invitrogen, Carlsbad, USA) by *Eco*RI and *Bam*HI digestion. pcDNA3-Myc-AChE was constructed by transferring human AChE to pcDNA3-Myc (derived from pcDNA3.0, Invitrogen) by *Eco*RI and *Bam*HI digestion. pEGFP-C1-AChE was constructed by transferring human AChE to pEGFP-C1 (Clontech) by *Bgl*II and *Eco*RI digestion. All the constructs were verified by DNA sequencing.

Yeast two-hybrid screening

Yeast two-hybrid screening was performed using a matchmaker GAL4 two-hybrid system 3 (Clontech) according to the manufacturer's instructions. A 159-bp fragment coding for 54 amino acids of the C-terminus of human AChE (AChE$_{C\text{-terminal}}$) was fused to the GAL4-DNA-binding domain of the pGBKT7 vector (Clontech). The human fetal brain matchmaker cDNA library (Clontech) was screened. To confirm the interaction, the rescued library plasmid of the positive clone was cotransfected along with the bait plasmid into yeast and analyzed by liquid culture assay with chlorophenol red-β-D-galactopyranoside as a substrate.

Cell culture, transfection, and drug treatment

HEK293T cells (Catalog, No. GNHu17) were cultured in DMEM supplemented with 10% fetal bovine serum at 37°C in a 5% CO_2 atmosphere. Torriglia's long-term culture method was employed to induce apoptosis in HEK392T cell [35]. Briefly, cells were maintained for up to 8 days without medium change at a density of 1×10^5 cells/ml. Then, the cells were transfected using a calcium-phosphate-mediated transfection method [36]. After transfection, 30 mg/ml cisplatin (Sigma, St. Louis, USA) in DMSO was added directly to the cell medium at the final concentration of 200 μM, and the cells were incubated for 24 h.

DNA-ladder-formation assay

The cells were disrupted in lysis buffer (5 mM Tris, 20 mM EDTA, pH 8.0) on ice for 30 min followed by centrifugation (16,000 *g*, 4°C, 20 min). The supernatant was collected and proteinase K (50 μg/ml) and RNase A (40 μg/ml) were added. After incubation for 1 h at 37°C, DNA was extracted by adding the same volume of phenol/chloroform. After shaking and centrifugation, the upper phase was collected. Then, one-tenth volume of 3 M sodium acetate (pH 5.2) and two volumes of ice-cold (−20°C) ethanol were added, and the samples were incubated overnight at −20°C. After centrifugation (16,000 *g*, 4°C, 30 min), the pellet was dissolved in TE buffer. DNA was analyzed in 1.5% agarose gel.

MTT staining

Cells were seeded into 96-well plates (1×10^4 cells/well) in triplicate and grown for 18 h. Then, the cells were exposed to various concentrations (50, 100, 200 μM) of cisplatin for 24 h. After 24-h cisplatin treatment, 20 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (0.5 mg/ml) was added along with 200 μl of medium into each well. The resulting cells were incubated for 3 h at 37°C, and the converted dye was solubilized with acidic isopropanol (0.04–0.1 M HCl in absolute isopropanol). The absorbance of the converted dye was measured at 570 nm, with background subtraction at 630 nm.

Co-immunoprecipitation and immunoblotting

Cells were lysed in lysis buffer (150 mM NaCl, 25 mM Tris–HCl, pH 7.4, 0.2 mM PMSF, 2 mM EDTA, 0.5% Triton X-100) on ice for 30 min followed by centrifugation (12,000 *g*, 4°C, 30 min). Since AChE is expressed during ischemia/reperfusion (I/R)-induced apoptosis *in vivo* [37], the kidney tissues of male Sprague–Dawley rats used as ischemia/reperfusion injury models [37] were homogenized in lysis buffer with complete protease inhibitors mixture (Roche Applied Science) for 30 min on ice. Then, 1 mg of the total lysate was precleared with protein A-Sepharose for 1 h at 4°C and coimmunoprecipitated with 2 μg of antibody for 2 h. Then, protein A-Sepharose was added, and the mixture was incubated for an additional 1 h at 4°C. The beads were washed three times with the lysis buffer. Immunoprecipitations were performed using the following antibodies: anti-Myc antibody (Cell Signalling, #2276), anti-AChE antibody (E70, the antibody against the N-terminal peptide GPVSAFLGIPFAEC was prepared in the rabbit [37]), and anti-rabbit IgG antibody (Pierce, No. 31210). The immunoblots were processed as described previously [8]. The primary antibodies used were as follows: anti-RanBPM antibody (NB100-1281; Novus Biologicals) (1:1000), anti-AChE antibody (the antibody against AChE was prepared in the rabbit [37]) (1:1000), anti-Myc antibody (1:5000), anti-AChE antibody (1:1000), anti-GFP antibody (sc-9996; Santa Cruz) (1:1000), anti-HA antibody (sc-805; Santa Cruz) (1:500), anti-caspase 3 antibody (Cell Signalling, #9662) (1:500), anti-histone 3 antibody (ab1791; ABCAM) (1:3000), anti-tubulin antibody (T6074; Sigma) (1:5000), anti-actin antibody (A5441; Sigma) (1:5000). The blots were developed using the ECL chemiluminescence kit (Amersham Biosciences).

Cytoplasmic and nuclear protein extraction

The nuclear extract and the cytoplasmic fraction of the cells were obtained by using NE-PER™ nuclear and cytoplasmic extraction kit (Pierce, #78833).

Immunofluorescence

The adherent cells were washed with cold phosphate-buffered saline (PBS) twice, and fixed in a paraformaldehyde solution (4% in PBS, pH 7.4) for 30 min at 4°C. The cultures were subsequently washed twice with PBS, treated with permeabilization solution (0.1% Triton X-100 in 0.1% sodium citrate) on ice for 2 min, and incubated in blocking buffer (3% BSA in TBST) for 1 h. Then, the cells were incubated with the anti-Myc antibody (1:100) in TBST containing 3% BSA overnight at 4°C. After incubation, the cells were washed and incubated with the secondary antibody Rhodamine-conjugated anti-mouse IgG-R (Santa Cruz) (1:100) in the dark at 37°C for 1 h. After washing, the cells were incubated in 2 μg/ml Hoechst33258 for 10 min at 37°C in the dark, and then observed under a fluorescence microscope.

Semi-quantitative RT–PCR

Total RNA was extracted from the cells or rat tissues using TRIzol reagent (Invitrogen) and was reverse transcribed using M-MLV reverse transcriptase (RT) (Promega, Madison, USA). The resulting cDNAs were used as templates to amplify *RanBPM* (539 bp) (forward primer, 5′-CGCATCCAATACCAGCAGCC-3′; reverse primer, 5′-GGCACAGTACCCATGGTGGA-3′), *p73α* (229 bp) (forward primer, 5′-CATGGTCTCGGGGTCC-CACT-3′; reverse primer, 5′-CTGCTTCAGGTCCTGCA-GGC-3′), and *actin* (257 bp) (forward primer,

5′-CAACTCCATCATGAAGTGTGACG-3′; reverse primer, 5′-ACTCGTCATACTCCTGCTTGC-3′) by PCR.

Results

Identification of RanBPM as an interacting partner for AChE

In order to identify the proteins interacting with AChE during apoptosis, we fused a fragment corresponding to C-terminal of AChE into the pGBKT7 vector. Since yeast clones transformed with the bait plasmid could only survive on SD/-Trp (synthetic dropout medium lacking Trp) [**Fig. 1(A)**], the bait had no self-transcription activity and was used to screen the human fetal brain cDNA library. Approximately 2.16×10^6 clones were screened [**Fig. 1(B)**]. This produced 10 independent clones reflecting partner protein interactions. According to sequencing and BLAST analysis, six of them could locate in brain and muscle, and two of them mainly in brain. On the subcellular location, one of the partners segregates to the nucleus and the others distribute in cytoplasma or membrane. Sequence analysis revealed that two of positive clones (clones A and C6) encoded partial sequences of RanBPM. The sequence from the C6 clone corresponded to amino acids 135–729 of RanBPM, whereas that from the A clone corresponded to amino acids 20–729 of RanBPM [**Fig. 1(C)**]. The interaction between $AChE_{C\text{-terminal}}$ and RanBPM was reconfirmed by liquid β-galactosidase assay [**Fig. 1(D)**]. The result of liquid β-galactosidase showed that comparing with the positive control, the affinity is weaker, but stronger than the negative control.

Coimmunoprecipitation analysis was performed to confirm the interaction between RanBPM and $AChE_{C\text{-terminal}}$. As shown in **Fig. 1(E)**, HA-RanBPM coimmunoprecipitated with Myc-$AChE_{C\text{-terminal}}$, as well as with Myc-AChE [**Fig. 1(F)**].

Since RanBPM is conserved in mammals [34], we used the kidney tissues of a rat model of ischemia/reperfusion injury to investigate the interaction between endogenous RanBPM and endogenous AChE. Endogenous RanBPM was found to be able to coimmunoprecipitate with endogenous AChE, in comparison with the findings for an unrelated control antibody

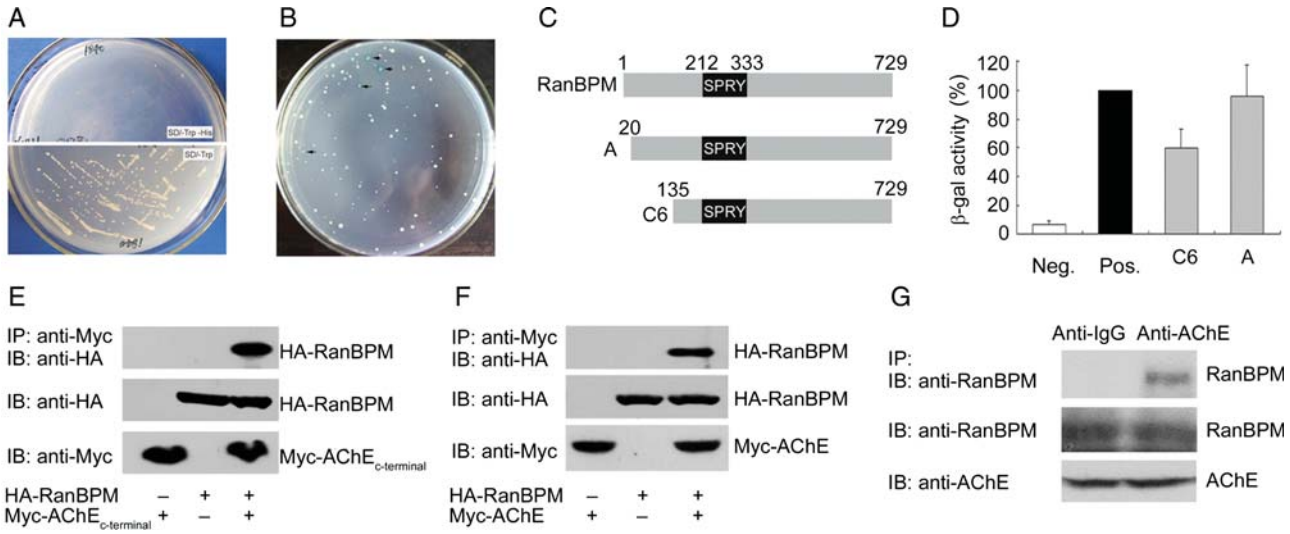

Figure 1 AChE interacts with RanBPM (A) The self-expression assay of the bait. AH109/pGBKT7-$AChE_{C\text{-terminal}}$ cells were cultured on SD/-Trp plate or SD/-Trp/-His plates. (B) Library screening. AH109 clones transfected with pGBKT7-$AChE_{C\text{-terminal}}$ and human fetal brain library were cultured on SD/-Ade/-His/-Leu/-Trp/X-α-gal plates. The arrows indicate the positive clones (blue clones). (C) The scheme of the two positive clones (A and C6). The putative SPRY domain (amino acids 212–333) is indicated. (D) Clones A and C6 showed strong lacZ reporter activation. Pos., a clone consisting of BD-p53 and AD-SV40 T-antigen was used as the positive control. Neg., a combination of BD-laminC and AD-SV40 T-antigen was used as the negative control; β-galactosidase activity is expressed as a percentage of the positive control. The data are represented as the mean ± SD of three independent transformations. (E,F) HEK293T cells transfected with the indicated expression plasmid for 24 h; the whole-cell lysates were immunoprecipitated with anti-Myc antibody and analyzed by immunoblotting with anti-HA antibody (top panel). Separate aliquots of the lysates were immunoblotted with anti-HA (middle panel) or anti-Myc (bottom panel) to confirm the expression of HA-RanBPM or Myc-$AChE_{C\text{-terminal}}$ (or Myc-AChE), respectively. (G) Kidney tissues from the rat model of ischemia/reperfusion injury were immunoprecipitated using the anti-AChE antibody (E70), and analyzed by immunoblotting with the anti-RanBPM antibody (top panel). Separate aliquots of the lysates were immunoblotted with anti-RanBPM (middle panel) or anti-AChE (E70) (bottom panel) to confirm the expression of RanBPM or AChE, respectively, in the rat kidney tissues.

[**Fig. 1(G)**]. Thus, RanBPM was identified as a protein partner of AChE-S.

Ubiquitous expression of RanBPM in normal rat tissues and human cell lines

The RanBPM mRNA was extracted from different rat tissues (brain, kidney, heart, liver, lung, ovary, pancreas, muscle, spleen, testis, uterus, and epididymis) or human cell lines (HeLa, HepG2, K562, 7721, and HEK293T) and detected by RT–PCR. As shown in **Fig. 2**, RanBPM was widely expressed in various rat tissues and human cell lines.

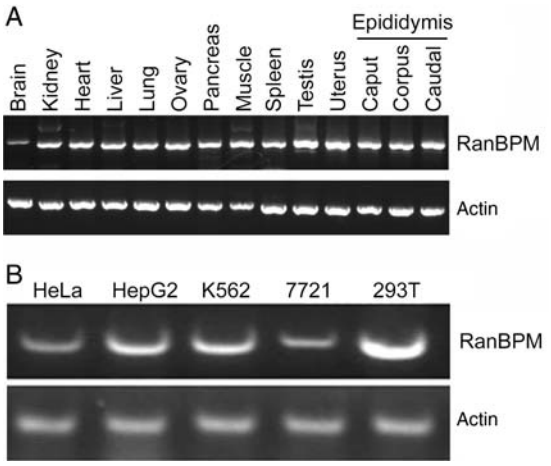

Figure 2 Expression of RanBPM in normal rat tissues and human cell lines (A) Total mRNAs from rat tissues. (B) Total mRNAs from human cell lines.

Subcellular distribution of RanBPM and AChE

To examine the subcellular location of RanBPM and AChE, HEK293T cells were transfected with the expression plasmids mentioned in a previous section. As shown in **Fig. 3(A,B)**, HA-RanBPM and Myc-AChE$_{C\text{-terminal}}$ (or Myc-AChE) were detected in the cytoplasm. The results of the indirect immunofluorescent staining, which showed that Myc-RanBPM and GFP-AChE were clearly co-localized in the cytoplasm [**Fig. 3(C)**], were consistent with those of the western blot analysis.

Subcellular distribution of RanBPM and AChE after cisplatin treatment

HEK293T cells were incubated with various doses of cisplatin, and their viability was determined using the MTT assay. The results revealed that cisplatin at concentration from 50 to 200 μM caused moderate cytotoxicity to cultured HEK293T cells [**Fig. 4(A)**]. The total mRNA was extracted from HEK293T cells that treated with or without cisplatin, and detected by RT–PCR. As shown in **Fig. 4(B)**, compared with HEK293T cells without

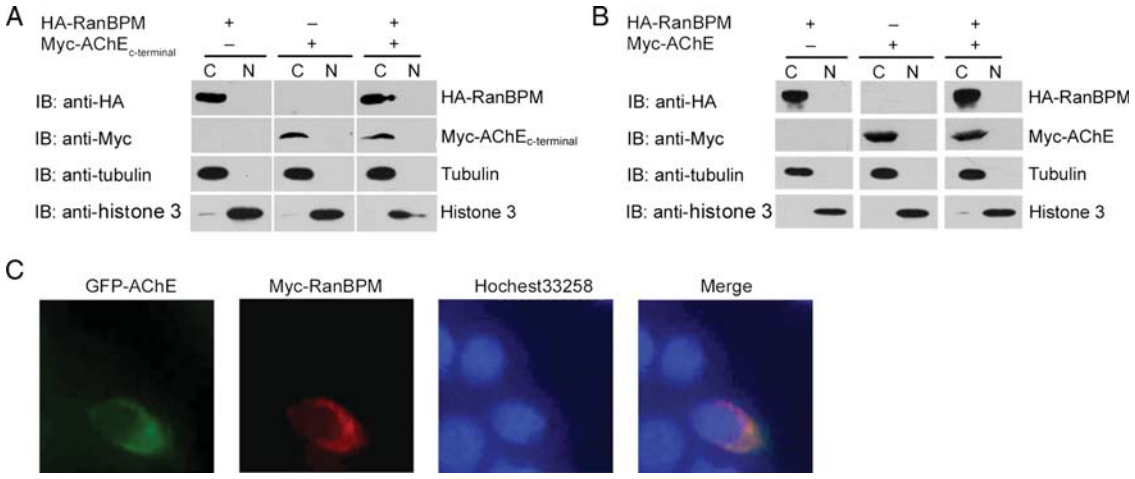

Figure 3 Subcellular distribution of RanBPM and AChE (A,B) Fractionation of HEK293T cell extracts. HEK293T cells were transfected with the indicated expression plasmids. At 24 h after transfection, the cells were fractionated into nuclear (N) and cytoplasmic (C) fractions and analyzed directly by immunoblotting with the anti-HA (first panel) or anti-Myc antibody (second panel). The purity of the nuclear and cytoplasmic fractions was examined by immunoblotting with anti-histone 3 (fourth panel) and anti-α-tubulin antibody (third panel), respectively. (C) Determination of co-localization of AChE and RanBPM by immunofluorescence. HEK293T cells were transfected with Myc-RanBPM and GFP-AChE. At 24 h after transfection, the cells were fixed and incubated with anti-Myc (red), followed by incubation with Rhodamine-conjugated secondary antibodies. The cells were examined by fluorescence microscopy.

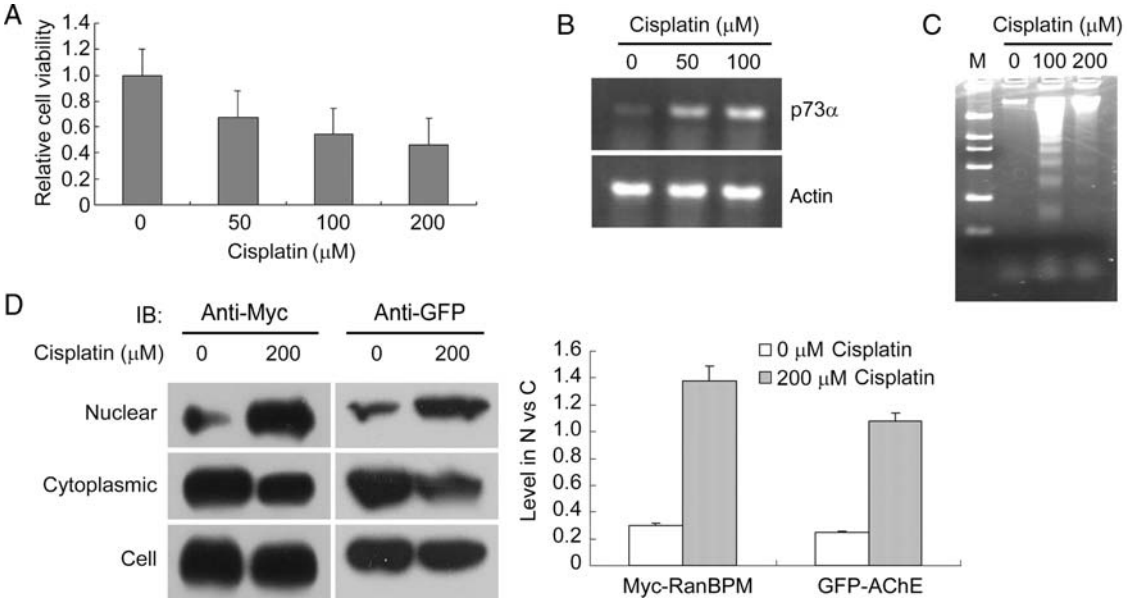

Figure 4 Subcellular distribution of RanBPM and AChE after cisplatin treatment (A) HEK293T cell viability was assessed by MTT after treatment with different concentrations of cisplatin for 24 h. The graph shows the mean ± SE of three independent experiments. (B) The transcription level of *p73α* gene detected by RT–PCR. HEK293T cells were treated with different concentrations of cisplatin for 24 h and then total mRNA was extracted for RT–PCR. (C) Cisplatin of 100 μM or higher induced DNA fragmentation. (D) The amount of RanBPM and AChE in the nuclear fractions increased after treatment with cisplatin. After cotransfection with pCMV-Myc-RanBPM and pEGFP-C1-AChE for 24 h, HEK293T cells were treated with the indicated concentrations of cisplatin for another 24 h. Then, the cells were fractionated into nuclear (N) and cytoplasmic (C) fractions. The cell fractions and the whole-cell lysates were immunoblotted with anti-Myc (left line) or anti-GFP (light line). The graph shows the densitometric values of the proteins in the cytoplasm versus those in the nuclei.

cisplatin treatment, the transcription level of *p73α* gene was increased after cisplatin treatment. One hallmark of apoptosis is the formation of DNA ladders. Therefore, we investigated this aspect. It was found that cisplatin at concentrations of 100 and 200 μM caused fragmentation of nuclear DNA [**Fig. 4(C)**]. Since cisplatin at concentrations of 100 and 200 μM elicited a marked decrease in cell survival, we selected the 200-μM cisplatin as the apoptosis inducer.

HEK293T cells co-expressing GFP-AChE and Myc-RanBPM were treated with or without cisplatin and fractionated into nuclear and cytoplasmic fractions. At the same levels of exogenous protein expression, the amounts of Myc-RanBPM and GFP-AChE in the nuclear fractions of the cells that had undergone cisplatin treatment were both clearly higher than those in the cells that did not undergo cisplatin treatment [**Fig. 4(D)**]. In conclusion, the amounts of AChE and RanBPM in the nuclear fractions increased after cisplatin treatment.

Nuclear distribution of endogenous RanBPM and AChE after long-term culture

HEK293T cells were incubated for indicated time without medium change, and the occurrence of apoptosis was determined by cleaved caspase 3. Our results revealed that cleaved caspase 3 could be detected at the third day and was obvious at the seventh day [**Fig. 5(A)**]. HEK293T cells in long-term culture were fractionated into nuclear fractions. As

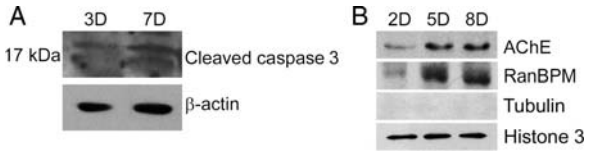

Figure 5 Nuclear distribution of endogenous RanBPM and AChE after long-term culture (A) Cleaved caspase 3 was detected in long-term cultured HEK293T cells. HEK293T cells were cultured without medium changed for 3 days (3D) or 7 days (7D), and then the whole-cell lysates were immunoblotted with anti-capspase 3, or anti-β-actin antibody. (B) The amount of endogenous RanBPM and AChE in the nuclear fractions was increased after long-term culture. HEK293T cells were cultured without medium changed for 2 days (2D), 5 days (5D) or 8 days (8D), and then the nuclear fractions were immunoblotted with anti-AChE (first panel) or anti-RanBPM antibody (second panel, NB 100–1281). The purity of the nuclear and cytoplasmic fractions was examined by immunoblotting with anti-α-tubulin (third panel) or anti-histone 3 antibody (fourth panel), respectively.

shown in **Fig. 5(B)**, the amounts of endogenous RanBPM and endogenous AChE in the nuclear fractions of the cells that had undergone apoptosis were both clearly higher than those in the cells that did not undergo apoptosis.

Discussion

AChE-T has a 40- or 41-aa C-terminal peptide that is extremely well conserved in all vertebrates. It has been reported that a synthesized AChE-peptide from the C-terminal region induced neuronal cell death [19]. To identify the interacting proteins that are involved in apoptosis mediated by AChE-S, we employed the human AChE-S C terminus, which had no self-transcription activity [**Fig. 1(A)**], to screen the human fetal brain library. Here, we identified RanBPM as an AChE-interacting protein (**Fig. 1**).

RanBPM is a highly conserved protein (**Fig. 3**), which has already been shown in other studies. RanBPM is ubiquitously expressed in all analyzed tissues, including different brain regions, and cell lines [34,38]. Within the brain, RanBPM occurs in the cortex, cerebellum, amygdala, hippocampus, and thalamus [39,40].

On the subcellular level, RanBPM can be found in the cytoplasm, the nucleus and the plasma membrane [34,41]; however, several studies have shown that endogenous RanBPM is primarily localized in the cytoplasm and membranes of the cells and does not appear to be present in the nucleus [29,42,43]. It can be speculated that there are different RanBPM complexes in different cellular environments such as the cell plasma membrane, cytosol, and nucleus. RanBPM was originally identified as a Ran GTPase-binding protein [26]. Unlike most other identified Ran-binding proteins, RanBPM does not contain the consensus Ran-binding domain [33], a finding that is consistent with its lack of involvement in nuclear-cytoplasmic transport [34]. The results of analysis with the bioinformatics program PSORTII [44] suggest that RanBPM shows no nuclear-localization signals (NLSs) and predict that RanBPM has a higher possibility of being cytoplasmic rather than nuclear, since the basic residue content calculated by PSORT was 7.7%. In our study, separate or synchronous expression of AChE and RanBPM corresponded to the detection of the two proteins in the cytoplasm (**Fig. 4**).

Cisplatin is a widely used chemotherapeutic agent that is generally recognized as a DNA-damaging drug. AChE was induced in various cell types by different apoptotic stimuli [8,45]. In HEK293T cells, p53 is inactivated by the SV40 T antigen. Despite the lack of p53 response, HEK293T cells succumbed to cisplatin treatment [46]. However, as shown in **Fig. 4(A,C)**, a higher concentration of cisplatin was needed to induce this response.

RanBPM can stabilize the levels of p73α which can modulate the cellular proteins/pathways that specifically regulate nuclear import and export of RanBPM [43]. p73 is an identified p53-related nuclear transcription factor that promotes cell arrest or apoptosis [47], and it is significantly induced at the protein level in response to certain genotoxic stresses, including cisplatin treatment. This induction is mediated by a nuclear non-receptor tyrosine kinase c-Abl [48–50]. The transcription level of *p73α* gene was increased after cisplatin treatment [**Fig. 4(B)**]. As shown in **Fig. 4(D)**, the quantity of RanBPM in the nuclear fraction increased after cisplatin treatment.

Our previous studies using morphologic methods showed that the AChE protein was present in the cytoplasm at the initiation of apoptosis and in the nucleus or apoptotic bodies upon commitment to cell death [8,51]. In living normal rat kidney (AChE over-expressing) cells, AChE protein was detected in the cytoplasm, but not in the nucleus; however, in the apoptotic cells, it was detected both in the cytoplasm and nucleus [9]. Coincident with the data from our previous studies, the quantity of AChE in the nuclear fraction increased after cisplatin treatment [**Fig. 4(D)**].

Myc-RanBPM and GFP-AChE were expressed under the $P_{CMV\ IE}$ promoter (cytomegalovirus immediate-early promoter) in order to check the subcellular localization of the two proteins during apoptosis. The activity of the $P_{CMV\ IE}$ promoter was different during the differentiation of embryonic stem cells into neurons [52]. So it was possible that the activity of $P_{CMV\ IE}$ promoter could be different between normal cells and apoptotic cells, and apoptosis-induced increases of both Myc-RanBPM and GFP-AChE in nuclear fractions could likely reflect changes in the efficacy of the CMV promoter. To exclude the possibility influence of changes caused by the activity of $P_{CMV\ IE}$ promoter, we checked the endogenous expression of AChE and RanBPM in the nuclear fractions in long-term HEK293T cultures. As shown in **Fig. 5**, the amounts of endogenous AChE and endogenous RanBPM in the nuclear fractions were increased after long-term culture, which was coincident with the result from co-expressed exogenous Myc-RanBPM and GFP-AChE in HEK293T after cisplatin treatment [**Fig. 4(D)**].

The subcellular localization of AChE-S was investigated using the bioinformatics program PSORTII [44]. There were no NLSs for the AChE protein. AChE was considered to have a higher possibility of being cytoplasmic than nuclear, since the basic-residue content calculated by PSORT was 8.6%. A protein without its own NLS may enter the nucleus via cotransport with another protein that has an NLS. Since both AChE and RanBPM have no NLSs, we deduced that there was no obviously direct relationship between their translocations from the cytoplasm to the nucleus during apoptosis (data not shown). As RanBPM was associated with several proteins and composed a large protein complex [41], it was possible that AChE and RanBPM composed a large protein complex with some protein that has an NLS.

In conclusion, RanBPM is an AChE-interacting protein that enters into the nucleus during apoptosis, similar to the translocation observed in case of AChE.

Acknowledgements

The authors would like to thank Hualin Wang, Detian Yuan, Wei Wei, and Dr Luolan Qian for their helpful advice and technical assistance.

Funding

This work was supported by the grants from the Major State Basic Research Development Program of China (973 Program) (No. 2007CB947901) and the Third Phase Creative Program of the Chinese Academy of Sciences (No. KSCX1-YW-R-13).

References

1 Massoulie J and Bon S. The molecular forms of cholinesterase and acetylcholinesterase in vertebrates. Annu Rev Neurosci 1982, 5: 57–106.
2 Li Y, Camp S and Taylor P. Tissue-specific expression and alternative mRNA processing of the mammalian acetylcholinesterase gene. J Biol Chem 1993, 268: 5790–5797.
3 Krejci E, Legay C, Thomine S, Sketelj J and Massoulie J. Differences in expression of acetylcholinesterase and collagen Q control the distribution and oligomerization of the collagen-tailed forms in fast and slow muscles. J Neurosci 1999, 19: 10672–10679.
4 Massoulie J, Pezzementi L, Bon S, Krejci E and Vallette FM. Molecular and cellular biology of cholinesterases. Prog Neurobiol 1993, 41: 31–91.
5 Soreq H and Seidman S. Acetylcholinesterase—new roles for an old actor. Nat Rev Neurosci 2001, 2: 294–302.
6 Paraoanu LE and Layer PG. Acetylcholinesterase in cell adhesion, neurite growth and network formation. FEBS J 2008, 275: 618–624.
7 Inestrosa NC, Dinamarca MC and Alvarez A. Amyloid-cholinesterase interactions. Implications for Alzheimer's disease. FEBS J 2008, 275: 625–632.
8 Zhang XJ, Yang L, Zhao Q, Caen JP, He HY, Jin QH and Guo LH, *et al*. Induction of acetylcholinesterase expression during apoptosis in various cell types. Cell Death Differ 2002, 9: 790–800.
9 Jin QH, He HY, Shi YF, Lu H and Zhang XJ. Overexpression of acetylcholinesterase inhibited cell proliferation and promoted apoptosis in NRK cells. Acta Pharmacol Sin 2004, 25: 1013–1021.
10 Park SE, Kim ND and Yoo YH. Acetylcholinesterase plays a pivotal role in apoptosome formation. Cancer Res 2004, 64: 2652–2655.
11 Johnson G and Moore SW. Human acetylcholinesterase binds to mouse laminin-1 and human collagen IV by an electrostatic mechanism at the peripheral anionic site. Neurosci Lett 2003, 337: 37–40.
12 Paraoanu LE and Layer PG. Mouse acetylcholinesterase interacts in yeast with the extracellular matrix component laminin-1β. FEBS Lett 2004, 576: 161–164.
13 Johnson G, Swart C and Moore SW. Interaction of acetylcholinesterase with the G4 domain of the laminin α1-chain. Biochem J 2008, 411: 507–514.
14 Greenfield SA, Day T, Mann EO and Bermudez I. A novel peptide modulates α7 nicotinic receptor responses: implications for a possible trophic-toxic mechanism within the brain. J Neurochem 2004, 90: 325–331.
15 Birikh KR, Sklan EH, Shoham S and Soreq H. Interaction of 'read-through' acetylcholinesterase with RACK1 and PKCβ II correlates with intensified fear-induced conflict behavior. Proc Natl Acad Sci USA 2003, 100: 283–288.
16 Perry C, Pick M, Podoly E, Gilboa-Geffen A, Zimmerman G, Sklan EH and Ben-Shaul Y, *et al*. Acetylcholinesterase/C terminal binding protein interactions modify Ikaros functions, causing T lymphopenia. Leukemia 2007, 21: 1472–1480.
17 De Ferrari GV, Canales MA, Shin I, Weiner LM, Silman I and Inestrosa NC. A structural motif of acetylcholinesterase that promotes amyloid β-peptide fibril formation. Biochemistry 2001, 40: 10447–10457.
18 Perris R and Perissinotto D. Role of the extracellular matrix during neural crest cell migration. Mech Dev 2000, 95: 3–21.
19 Day T and Greenfield SA. A peptide derived from acetylcholinesterase induces neuronal cell death: characterisation of possible mechanisms. Exp Brain Res 2003, 153: 334–342.
20 Day T and Greenfield SA. Bioactivity of a peptide derived from acetylcholinesterase in hippocampal organotypic cultures. Exp Brain Res 2004, 155: 500–508.
21 Mor I, Sklan EH, Podoly E, Pick M, Kirschner M, Yogev L and Bar-Sheshet Itach S, *et al*. Acetylcholinesterase-R increases germ cell apoptosis but enhances sperm motility. J Cell Mol Med 2008, 12: 479–495.
22 Mor I, Bruck T, Greenberg D, Berson A, Schreiber L, Grisaru D and Soreq H. Alternate AChE-R variants facilitate cellular metabolic activity and resistance to genotoxic stress through enolase and RACK1 interactions. Chem Biol Interact 2008, 175: 11–21.
23 Johnson G and Moore SW. The adhesion function on acetylcholinesterase is located at the peripheral anionic site. Biochem Biophys Res Commun 1999, 258: 758–762.
24 Johnson G and Moore SW. Cholinesterases modulate cell adhesion in human neuroblastoma cells *in vitro*. Int J Dev Neurosci 2000, 18: 781–790.

25 Mack A and Robitzki A. The key role of butyrylcholinesterase during neurogenesis and neural disorders: an antisense-5′butyrylcholinesterase-DNA study. Prog Neurobiol 2000, 60: 607–628.
26 Nakamura M, Masuda H, Horii J, Kuma K, Yokoyama N, Ohba T and Nishitani H, *et al*. When overexpressed, a novel centrosomal protein, RanBPM, causes ectopic microtubule nucleation similar to γ-tubulin. J Cell Biol 1998, 143: 1041–1052.
27 Wang D, Li Z, Messing EM and Wu G. Activation of Ras/Erk pathway by a novel MET-interacting protein RanBPM. J Biol Chem 2002, 277: 36216–36222.
28 Hafizi S, Gustafsson A, Stenhoff J and Dahlback B. The Ran binding protein RanBPM interacts with Axl and Sky receptor tyrosine kinases. Int J Biochem Cell Biol 2005, 37: 2344–2356.
29 Denti S, Sirri A, Cheli A, Rogge L, Innamorati G, Putignano S and Fabbri M, *et al*. RanBPM is a phosphoprotein that associates with the plasma membrane and interacts with the integrin LFA-1. J Biol Chem 2004, 279: 13027–13034.
30 Cheng L, Lemmon S and Lemmon V. RanBPM is an L1-interacting protein that regulates L1-mediated mitogen-activated protein kinase activation. J Neurochem 2005, 94: 1102–1110.
31 Rao MA, Cheng H, Quayle AN, Nishitani H, Nelson CC and Rennie PS. RanBPM, a nuclear protein that interacts with and regulates transcriptional activity of androgen receptor and glucocorticoid receptor. J Biol Chem 2002, 277: 48020–48027.
32 Brunkhorst A, Karlen M, Shi J, Mikolajczyk M, Nelson MA, Metsis M and Hermanson O. A specific role for the TFIID subunit TAF4 and RanBPM in neural progenitor differentiation. Mol Cell Neurosci 2005, 29: 250–258.
33 Beddow AL, Richards SA, Orem NR and Macara IG. The Ran/TC4 GTPase-binding domain: identification by expression cloning and characterization of a conserved sequence motif. Proc Natl Acad Sci USA 1995, 92: 3328–3332.
34 Nishitani H, Hirose E, Uchimura Y, Nakamura M, Umeda M, Nishii K and Mori N, *et al*. Full-sized RanBPM cDNA encodes a protein possessing a long stretch of proline and glutamine within the N-terminal region, comprising a large protein complex. Gene 2001, 272: 25–33.
35 Torriglia A, Negri C, Chaudun E, Prosperi E, Courtois Y, Counis MF and Scovassi AI. Differential involvement of DNases in HeLa cell apoptosis induced by etoposide and long term-culture. Cell Death Differ 1999, 6: 234–244.
36 Jordan M, Schallhorn A and Wurm FM. Transfecting mammalian cells: optimization of critical parameters affecting calcium-phosphate precipitate formation. Nucleic Acids Res 1996, 24: 596–601.
37 Su W, Wu J, Ye WY and Zhang XJ. A monoclonal antibody against synaptic AChE: a useful tool for detecting apoptotic cells. Chem Biol Interact 2008, 175: 101–107.
38 Emberley ED, Gietz RD, Campbell JD, HayGlass KT, Murphy LC and Watson PH. RanBPM interacts with psoriasin *in vitro* and their expression correlates with specific clinical features *in vivo* in breast cancer. BMC Cancer 2002, 2: 28.
39 Poirier MB, Laflamme L and Langlois MF. Identification and characterization of RanBPM, a novel coactivator of thyroid hormone receptors. J Mol Endocrinol 2006, 36: 313–325.
40 Poirier MB, Brunelle M and Langlois MF. The effect of RanBPM on the regulation of the hypothalamic-pituitary axis by thyroid hormone receptors is isoform-specific. Biochem Biophys Res Commun 2007, 362: 516–521.
41 Kobayashi N, Yang J, Ueda A, Suzuki T, Tomaru K, Takeno M and Okuda K, *et al*. RanBPM, Muskelin, p48EMLP, p44CTLH, and the armadillo-repeat proteins ARMC8alpha and ARMC8beta are components of the CTLH complex. Gene 2007, 396: 236–247.
42 Zou Y, Lim S, Lee K, Deng X and Friedman E. Serine/threonine kinase Mirk/Dyrk1B is an inhibitor of epithelial cell migration and is negatively regulated by the Met adaptor Ran-binding protein M. J Biol Chem 2003, 278: 49573–49581.
43 Kramer S, Ozaki T, Miyazaki K, Kato C, Hanamoto T and Nakagawara A. Protein stability and function of p73 are modulated by a physical interaction with RanBPM in mammalian cultured cells. Oncogene 2005, 24: 938–944.
44 Nakai K and Horton P. PSORT: a program for detecting sorting signals in proteins and predicting their subcellular localization. Trends Biochem Sci 1999, 24: 34–36.
45 Steinritz D, Emmler J, Hintz M, Worek F, Kreppel H, Szinicz L and Kehe K. Apoptosis in sulfur mustard treated A549 cell cultures. Life Sci 2007, 80: 2199–2201.
46 Bae IH, Kang SW, Yoon SH and Um HD. Cellular components involved in the cell death induced by cisplatin in the absence of p53 activation. Oncol Rep 2006, 15: 1175–1180.
47 Kaghad M, Bonnet H, Yang A, Creancier L, Biscan JC, Valent A and Minty A, *et al*. Monoallelically expressed gene related to p53 at 1p36, a region frequently deleted in neuroblastoma and other human cancers. Cell 1997, 90: 809–819.
48 Agami R, Blandino G, Oren M and Shaul Y. Interaction of c-Abl and p73α and their collaboration to induce apoptosis. Nature 1999, 399: 809–813.
49 Gong JG, Costanzo A, Yang HQ, Melino G, Kaelin WG, Jnr, Levrero M and Wang JY. The tyrosine kinase c-Abl regulates p73 in apoptotic response to cisplatin-induced DNA damage. Nature 1999, 399: 806–809.
50 Yuan ZM, Shioya H, Ishiko T, Sun X, Gu J, Huang YY and Lu H, *et al*. p73 is regulated by tyrosine kinase c-Abl in the apoptotic response to DNA damage. Nature 1999, 399: 814–817.
51 Yang L, He HY and Zhang XJ. Increased expression of intranuclear AChE involved in apoptosis of SK-N-SH cells. Neurosci Res 2002, 42: 261–268.
52 Bagchi B, Kumar M and Mani S. CMV promotor activity during ES cell differentiation: potential insight into embryonic stem cell differentiation. Cell Biol Int 2006, 30: 505–513.