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G protein β2 subunit interacts directly with neuropathy target esterase and regulates its activity

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Abstract

Neuropathy target esterase (NTE) was identified as the primary target of organophosphate compounds that cause a delayed neuropathy with degeneration of nerve axons. NTE is a novel phospholipase B anchored to the cytoplasmic face of endoplasmic reticulum and essential for embryonic and nervous development. However, little is known about the regulation of NTE. A human fetal brain cDNA library was screened for proteins that interact with NTE, Gβ2 and Gβ2-like I subunits were found to be able to bind the C-terminal of NTE in yeast. The interaction of Gβ2 and NTE was confirmed by *in vivo* co-immunoprecipitation analysis in COS7 cells. Furthermore, depletion of Gβ2 by RNA interference down regulated the activity of NTE but not its expression level. In addition, the activity of NTE was down regulated by the G protein signal pathway influencing factor, pertussis toxin, treatment *in vivo*. These findings suggest that Gβ2 may play a significant role in maintaining the activity of NTE.

Keywords: Neuropathy target esterase; Yeast two-hybrid; Signal transduction; G protein beta-2 subunit; Mammalian cell

1. Introduction

Neuropathy target esterase (NTE) was identified as the primary target of organophosphorus compounds (OP) that cause a delayed paralyzing syndrome with degeneration of nerve axons (Johnson, 1982). Although the inhibition and subsequent aging of NTE has been proposed to be an initiating event in OP-induced delayed neuropathy (OPIDN), the events that occur between NTE inhibition and the appearance of clinical pathology are not completely understood (Glynn, 2005). As such, characterizing the molecular and cellular functions of NTE should elucidate the mechanism of OPIDN.

NTE is a polypeptide of 1327 amino acids and contains two domains, an N-terminal regulatory domain

Abbreviations: cAMP, cyclic AMP; DMEM, Dulbecco's modified Eagle's medium; EDTA, ethylenediaminetetraacetic acid; ECL, enhanced chemiluminescence; Gβ2, guanine nucleotide binding protein beta polypeptide 2; GFP, green fluorescent protein; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; NP40, Nonidet P-40; NTE, neuropathy target esterase; NEST, NTE esterase activity domain; PBS, phosphate-buffered saline; PI, propidium iodide; PV, phenyl valerate; RNAi, RNA interference; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; TM, transmembrane helix

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and a C-terminal catalytic domain, which is conserved in most organisms from bacteria to man (Lush et al., 1998). Within the N-terminal domain is a transmembrane segment (TM), which usually anchors NTE to the cytoplasmic face of the endoplasmic reticulum (ER) (Akassoglou et al., 2004; Li, Dinsdale, & Glynn, 2003). The NTE esterase domain (NEST) exists between amino acids 727–1216 of human NTE and reacts with both ester substrates and covalent inhibitors in a manner very similar to NTE (Atkins & Glynn, 2000). Within the NEST domain, serine^{966} and two aspartate residues, Asp^{960} and Asp^{1086}, are critical for NTE function. Previous observations indicated that NTE displayed potent lysophospholipase activity in mouse brain (Quistad, Barlow, Winrow, Sparks, & Casida, 2003). This activity has been further characterized to be responsible for converting phosphatidylcholine to glycerophosphocholine in mammalian cells and regulated phosphatidylcholine homeostasis in *Drosophila* (Muhlig-Versen et al., 2005; Zaccheo, Dinsdale, Meacock, & Glynn, 2004). In mice, complete inactivation of the NTE gene resulted in embryonic lethality due to placental failure and impaired vasculogenesis (Moser et al., 2004; Winrow et al., 2003), while mice with a brain-specific deletion of NTE exhibited neurodegeneration (Akassoglou et al., 2004). In adult *Drosophila*, loss of swiss cheese/NTE activity causes neuronal and glial death (Muhlig-Versen et al., 2005). Together, these data suggest that NTE is essential for embryonic and nervous development.

Although the character and function of NTE has been elucidated, little is known about the regulation of its activity. Potential regulation of NTE activity is suggested by the similarity of sequences within NTE's amino-terminal domain to the regulatory subunit of protein kinase A (Lush et al., 1998): the implication of this sequence homology is that NTE may be regulated directly by the binding of cyclic AMP (cAMP). Although using NTE constructs expressed in *E. coli*, binding of the radiolabelled nucleotide to purified recombinant the regulatory domain of NTE was failed to be detected (Dremier, Kopperud, Doskeland, Dumont, & Maenhaut, 2003), overexpression of all constructs of NTE containing the regulatory domain leads to aggregation of the recombinant polypeptide so that its normal biologically active conformation may not be attained (Glynn, 2005). NTE converts phosphatidylcholine to glycerophosphocholine in the CDP-choline pathway, so the most obvious regulator of NTE activity would be its substrate, phosphatidylcholine. More recently, Sec14p has been shown to interact functionally with NTE1, the homologous protein of NTE in yeast, to increase the rate of phosphatidylcholine deacylation to glycerophosphocholine in yeast (Murray & McMaster, 2005). BLAST searching revealed a number of mammalian proteins with homology to Sec14p or with Sec14p-like domains: among these the human caytaxin protein is particularly intriguing in the context of NTE (Glynn, 2005). In order to find out possible protein that regulate NTE activity, a human fetal neural brain cDNA library was amplified to screen host proteins that can interact with NTE. We demonstrate that G protein beta-2 (guanine nucleotide binding protein beta polypeptide 2, Gβ2) subunit interacts directly with NTE and silence of Gβ2 down-regulates the activity of NTE, which indicates Gβ2 plays a role in maintaining the activity of NTE and suggests there is a novel regulatory mechanism for NTE activity.

2. Materials and methods

2.1. Materials

The pNTE-GFP and D16 plasmids, which contain the full-length human NTE gene, were generous gifts from Dr. P. Glynn's Lab (Toxicology Unit, MRC, UK, Li et al., 2003). Stably expressing small interfering RNA (siRNA) SH-SY5Y cells with lowered NTE activity was generated in our lab (Chang, Chen, & Wu, 2005; Chang, Wu et al., 2005). Cell culture reagents were purchased from Gibco BRL (Grand Island, NY, USA) and the transfection reagent Lipofectamine 2000 was purchased from Invitrogen Life Technologies (Groningen, The Netherlands). Human NTE-specific antibody against peptides corresponding to amino acids 37–48/1316–1327 (VPKTPAPDGPRK/LPQEPPGSATDA) was generated by Shanghai Casarray Co. Ltd. (Shanghai, China). Human fetal brain cDNA library, mouse anti-Myc monoclonal antibody, Living Colors monoclonal antibody (JL-8) and goat anti-mouse IgG (Fc specific) peroxidase conjugate were obtained from Clontech (Palo Alto, CA, USA). Rabbit anti-Gβ2 polyclonal antibody (C-16), mouse anti-actin monoclonal antibody (C-2), mouse anti-rabbit IgG HRP and goat anti-chicken IgY-HRP were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Enhanced chemiluminescence (ECL) reagents were obtained from Pierce Biotechnology (Rockford, IL, USA). Mipafox and phenyl valerate (PV) were synthesized in our laboratory as described by Johnson (1982). Annexin V-FITC and propidium iodide (PI) were purchased from Bosai Co. Ltd. (Beijing, China). Paraoxon and pertussis toxin were purchased from Sigma (St. Louis, MO, USA).

2.2. Plasmid construction

The plasmid pGBKT7-NTE-C-Ter, which expresses a GAL4-NTE-C-Ter (amino acid 727–1327) fusion, was constructed as the "bait" vector by inserting a 5′*Eco*RI and 3′*Bam*HI fragment prepared by PCR using D16 plasmid as a template (forward primer 5′-GTGAATTCCTCACCAACCCAGCCAGCA-3′; reverse primer 5′-TTGGATCCTCAGGCATCTGTGGCTGA-3′), into pGBKT7 vector, which has a GAL4-binding domain (Clontech). PCR fragment and in-frame insertion was confirmed by sequencing with pGBKT7 forward and reverse primers. To construct a plasmid encoding NTE minus first 40 amino acids, which encode the N-terminal TM, we designed a forward primer (5′-TCAGAATTCCCAGCCCCGGATGGCCCC-3′) with an *Eco*RI site and paired it with the same reverse primer as the pGBKT7-NTE-C-Ter. These primers were used to amplify the NTE fragment from the clone D16. The fragment was then cloned into pGBKT7 vector to generate the plasmid pGBKT7-ΔTM-NTE. The full-length Gβ2 fragment was generated by RT-PCR from mRNA isolated from SH-SY5Y cells (Invitrogen One-Step RT-PCR Kit) and was certified by sequencing. Then full-length Gβ2 cDNA was cloned into the plasmid pGADT7, which has a GAL4-activating domain, to construct pGADT7-Gβ2. In order to express full-length Gβ2 subunit tagged with Myc peptide in mammalian cells, this fragment was also sub-cloned into the pCMV-Myc to generate pCMV-Myc-Gβ2 construct. All constructs were certified by sequencing.

2.3. Yeast two hybrid screening assay

A human fetal brain cDNA library in the activation domain vector pACT2 was amplified using the recommended protocol of the manufacturer. To identify proteins that bound to the C-terminal of NTE, the human fetal brain cDNA library in pACT2 was introduced into the yeast reporter strain AH109, bearing a plasmid expressing GAL4-NTE-C-Ter fusion protein. 3×10^6 transformants were screened on a high stringency selective synthetic medium (SD/-Ade/-His/-Leu/-Trp/X-α-gal), including 10 mM 3-amino-1,2,4-triazole to suppress background growth. The X-β-gal filter assay and *o*-nitrophenyl-D-galactopyranoside assay were used for determining β-galactosidase activity according to the recommended protocol of the manufacturer (Clontech). Plasmid DNA from strong LacZ phenotype colonies was isolated by the electroporation method and sequenced using a GAL4 AD sequencing primer after restriction analysis. Candidate plasmids were retested by cotransformation in AH109 with either pGBKT7-C-Ter or full pGBKT7-ΔTM-NTE.

2.4. Western blotting

Cells were rinsed three times with ice-cold phosphate-buffered saline (PBS) and lysed in a lysis buffer (50 mM Tris, pH 7.5, 300 mM NaCl, 5 mM EGTA, 1 mM EDTA, 0.5% Triton X-100, 0.5% NP40, 0.1 mM phenylmethylsulfonyl fluoride, and a 10 μg/ml final concentration of each of aprotinin, leupeptin, and pepstatin). The lysates were clarified by centrifugation at 10,000 × *g* for 10 min at 4 °C and the supernatant were collected for further analysis. Protein concentrations were determined by Coomassie brilliant blue staining using bovine serum albumin (BSA) as a standard (Bradford, 1976). The protein samples were fractionated by SDS-PAGE with a 4% stacking gel and 10% separating gel and transferred to Hybond ECL nitrocellulose membrane (Amersham Pharmacia Biotech). The membranes were blocked in TBST (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.1% Tween 20) containing 5% non-fat milk at room temperature for at least 1 h, and then incubated sequentially with primary antibody and HRP-conjugated secondary antibody, and then detected by standard ECL method.

2.5. Coimmunoprecipitation assays

For transfection experiments, $1–3 \times 10^3$ COS7 cells were transfected with 2.5 μg pCMV-Myc-Gβ2 and 2.5 μg either pNTE-GFP or pEGFP-N3 vector separately by Lipofectamine 2000. Two days later, the cells were rinsed three times with ice-cold PBS and lysed in the lysis buffer (50 mM Tris, pH 7.5, 1% NP40, 0.5% Na_3VO_4, 150 mM NaCl, 1 mM EDTA). For immunoprecipitation, the cell lysates were incubated with anti-GFP, anti-Gβ2 and negative control antibodies, respectively, and rotated at 4 °C for 3 h. Protein A/G Agarose (Santa Cruz Biotechnology) beads were used to immobilize antibody-bound proteins. Immunoprecipitates were washed three times with lysis buffer and resuspended in SDS-PAGE for immunoblotting analysis.

2.6. RNA interference (RNAi) experiments

To generate RNAi against Gβ2, two pairs of 64 bp DNA were annealed to a double-strand DNA and inserted into linearized pSUPER-neo vector (Oligoengine) and then digested with *Hin*dIII and *Bgl*II. The construct was verified by DNA sequencing and produced siRNA to target the 5′-CACTGACCCAGATCACAGC and 5′-CTGAGACAGGAGGCCGAGC mRNA of Gβ2

in mammalian cells, named siGβ2-a3 and siGβ2-b5, respectively. Mock DNA was constructed in the same vector in parallel. The above two constructs and mock plasmid were transfected in the SH-SY5Y cells by Lipofectamine 2000 reagent and selected with 500 μg/ml G418 for 3 weeks. Positive cell clones that down-regulated Gβ2 expression were selected by western blotting and maintained in DMEM medium containing 200 μg/ml G418.

2.7. NTE and acetylcholinesterase (AChE) assays

Cellular NTE activity was assayed in homogenate as the paraoxon-resistant and mipafox-sensitive hydrolysis of the PV. The PV hydrolase activity was assayed as described in our lab (Chang, Chen, et al., 2005; Chang, Wu, et al., 2005). AChE activity was assayed as described with the modified Ellman procedure (Gorun, Proinov, Baltesuc, Balaban, & Barzu, 1978). Concentration of protein was measured by Coomassie brilliant blue G250 binding assay (Bradford, 1976).

2.8. Flow cytometry measurement of apoptosis

Cell apoptosis was determined by using annexin V-FITC and PI double staining. Cells were harvested, washed twice with cold PBS and centrifuged at 1500 rpm for 4 min. 10^5 cells were resuspended in 200 μl of binding buffer (10 mM HEPES/NaOH, pH 7.4; 140 mM NaCl, 2.5 mM $CaCl_2$), 10 μl annexin V-FITC, and 5 μl of PI. Then samples were incubated for 15 min in dark at room temperature. At last 300 μl binding buffer was added to every tube; samples were then filtered and analyzed on flowcytometer (Becton Dickinson). In all, 10,000 events were counted for each sample.

2.9. Treatment with pertussis toxin (PTX)

SH-SY5Y cells were treated with PTX by addition of toxin to the culture media at a final concentration of 100 or 200 ng/ml. Control cells were seeded from the same stock of cells into identical vessels and batches of media not supplemented with PTX. After 10-h treatment, the expression of NTE and Gβ2 was detected with western blotting; the activities of NTE and AChE were assayed as described above. And the cell viability was also evaluated at the end of the PTX treatment experiment. Cell medium containing 0.5 mg/ml MTT was added to each well and incubated at 37 °C in 95% air/5% CO_2 for 4 h. The insoluble formazan formed was dissolved in DMSO, and the absorbance was measured in a spectrophotometer at 570 nm with a background reading of 660 nm.

2.10. Statistical analysis

Data were generally expressed as mean ± standard error values. Groups of data were compared by one-way ANOVA. A difference between means was considered significant at a value of $P < 0.05$.

3. Results

3.1. Isolation of proteins that interact with NTE in a yeast two-hybrid screen

A Gal4-based yeast two-hybrid system was used to identify proteins that interact with the NTE protein. A plasmid expressing a GAL-NTE-C-Ter fusion protein, which contains the NEST activating domain, was used to screen a human fetal brain cDNA expression library in the yeast strain AH109. Only about 20 strong positive clones had been obtained and sequenced from 3×10^6 transformants library screen. The associated cDNAs of strong positive clones were characterized by DNA sequencing (Table 1). Four positive clones were found to encode the β2 subunit of heterotrimeric G-protein from three different starting codons, while two clones were shown to encode identical Gβ2-like I cDNA. Further analysis revealed that all clones contain the same WD40 domains. The interaction between Gβ2/Gβ2-like I and NTE was confirmed in the yeast strain AH109 by cotransformation using the plasmids recovered from clone C3/C6 and either pGBKT7-C-Ter or pGBKT7-ΔTM-NTE. All clones expressed the LacZ phenotype, but the yeast transfected with C3/C6 and negative control pGBKT7-Lam did not express LacZ phenotype, confirming their interaction in the yeast (data not shown).

Table 1
Partial cDNAs recovered from positive clones selected by the plasmid pGBKT7-NTE-C-Ter based on GAL4 yeast-two hybrid system

cDNA	Identity[a]	Codons[b]
C3	Gβ2 (NM005273)	135–340
C7	Gβ2 (NM005273)	135–340
C11	Gβ2 (NM005273)	137–340
C5	Gβ2 (NM005273)	268–340
C6	Gβ2-like I (NM006098)	106–317
C14	Gβ2-like I (NM006098)	106–317

[a] GenBank™ accession numbers are given in parentheses.
[b] Residues encoded by each insert are shown. The same Gβ2-like I cDNA was identified in two independent transformants.

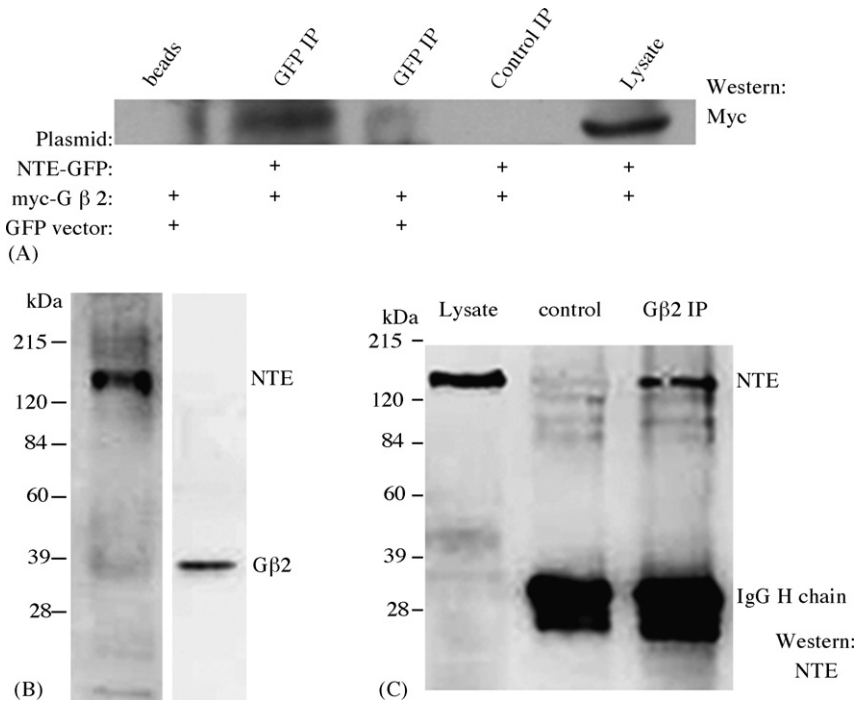

Fig. 1. *In vivo* association of NTE with Gβ2. (A) COS7 cells were transfected with mammalian expression plasmids encoding NTE-GFP or Myc-Gβ2. Anti-GFP (GFP-IP) or normal mouse IgG (control-IP), immunoprecipitates were analyzed by immunoblotting with anti-Myc. Plasmids used for cotransfections are listed below and immunoprecipitation with antibodies or beads alone are shown above. (B) SH-SY5Y cell lysates were resolved on SDS-PAGE, transferred to Hybond ECL nitrocellulose membrane, and analyzed by immunoblotting with anti-NTE or anti-Gβ2 antibodies. (C) SH-SY5Y cell lysates were immunoprecipitated either by anti-Gβ2 or control IgG (normal rabbit IgG), and the immunoprecipitated products were detected by immuno-blotting with anti-NTE.

A full characterization of Gβ2-like I protein is currently in progress.

3.2. Coimmunoprecipitation confirms the interaction of Gβ2 and NTE

To demonstrate the interaction between NTE and Gβ2 in a mammalian cell system, we expressed NTE-GFP and Myc-Gβ2 fusion proteins in COS7 cells by cotransfection with pNTE-GFP and pCMV-cMyc-Gβ2 construct. When NTE-GFP was immunoprecipitated from cell lysates with anti-GFP antibody, Myc-Gβ2 was also precipitated, as detected by the anti-Myc antibody (Fig. 1A). These data indicate that NTE binds to Gβ2 in mammalian cells. To determine whether endogenous NTE can form a complex with Gβ2, co-immunoprecipitation assays were performed with SH-SY5Y cell lysates. The expression of NTE and Gβ2 in SH-SY5Y cells was detected by western blotting with anti-Gβ2 and anti-NTE antibody, respectively, which showed the specificity of the two antibodies to the corresponding proteins (Fig. 1B). The Gβ2 was then immunoprecipitated from cell lysates with anti-Gβ2 antibody and NTE protein was precipitated too, which was also detected out by the anti-NTE antibody (Fig. 1C). These data revealed that anti-Gβ2, but not control antibody, could co-precipitate NTE with Gβ2, which further confirmed the physical interactions between NTE and Gβ2 proteins.

3.3. NTE activity is down-regulated by knockdown of Gβ2

In order to elucidate the regulatory role of Gβ2 in NTE activity, the effect of depletion of endogenous Gβ2 on NTE activity in SH-SY5Y neuroblastoma cells was investigated. Two stable expressing Gβ2-specific siRNAs cell clones were selected and named as siGβ2-a3 and siGβ2-b5, respectively. In siGβ2-a3 cells, the expression of Gβ2 was significantly suppressed compared to control cell, whereas no evident change in the level of Gβ2 in siGβ2-b5 (Fig. 2A). Then the activity of NTE *in situ* was assayed in the control cells, siGβ2-a3, and siGβ2-b5 cells. NTE activity of siGβ2-a3 cells was about 58% of control cells transfected with the empty pSUPER-neo vector. In contrast, there was no significant difference between NTE activity in siGβ2-b5 cells

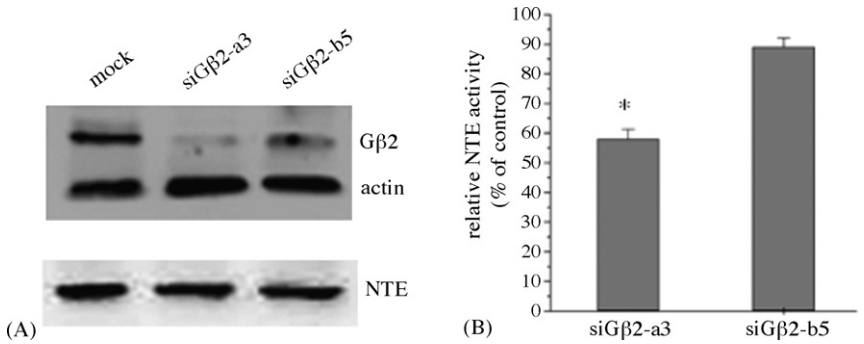

Fig. 2. Effect of silencing of Gβ2 expression on the level and activity of NTE in SH-SY5Y cells. (A) SH-SY5Y cells were transfected with siGβ2 and mock plasmid, then selected by addition of G418 for about 3 weeks. Extracts from the mono-cloned cells were analyzed by SDS-PAGE/immunoblot analysis with antibodies specific for Gβ2, NTE and actin. (B) Quantitative analysis of basal *in situ* NTE activity of cloned SH-SY5Y cells. Data are presented as a percentage of *in situ* NTE activity in mock cells. Results are mean ± S.E. (bars) from three independent tests. Statistical analysis of control cells vs. siGβ2 cells was carried out by ANOVA. $^{*}p < 0.05$, compared with controls, $n = 3$.

and that in control cells (Fig. 2B). However, the levels of NTE protein were not changed by the depletion of Gβ2 (Fig. 2A). Interestingly, siGβ2-a3 cells depleted of Gβ2 showed different morphology compared with control cells and appeared to be more susceptible to die in normal culture conditions (Fig. 3A and B). Apoptosis analysis showed that siGβ2-a3 cell has a higher apoptosis ratio compared with the control cell and the increased levels of apoptosis resulted from the depletion of Gβ2, but not NTE, for the reason that there was no difference of apoptosis between the control cells and the siNTE-cells with lowered NTE activity (Fig. 3C).

3.4. Effect of PTX treatment on the activity of NTE

Previous study indicated that PTX action appeared to be composed of two, temporally distinct, groups of effects: the early group of effects in the 6-h treatment was

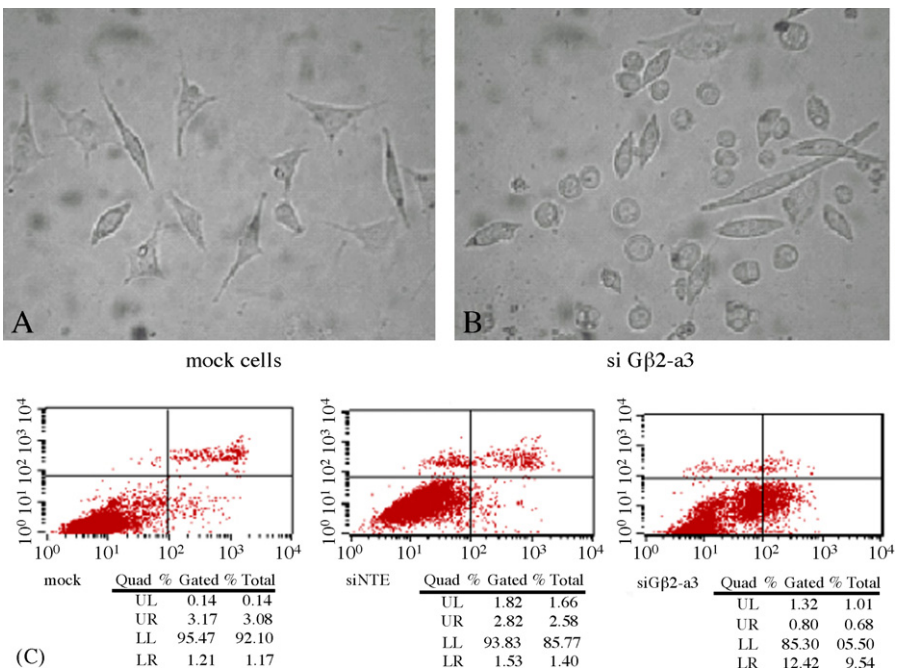

Fig. 3. Inhibition of Gβ2 expression altered cell morphology and resulted in apoptosis in SH-SY5Y cells. (A and B) The cell morphology of control cells (A) and siGβ2-a3 cells with inhibition of Gβ2 expression (B). (C) Apoptosis analysis of SH-SY5Y cells depleted of Gβ2, NTE protein or transfected with mock plasmid. Cells stained with annexin V-FITC and PI, then acquired for scan and analysis. Data are representative of triplicates of three independent experiments.

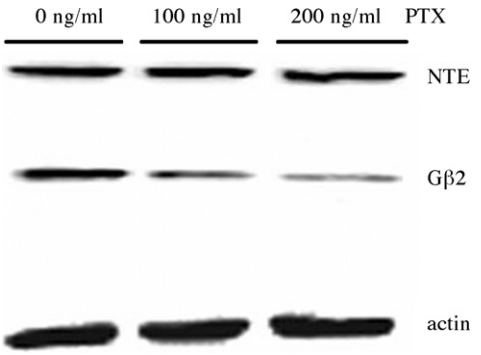

Fig. 4. Effect of pertussis toxin treatment on the expression of NTE and Gβ2 in SH-SY5Y cells. SH-SY5Y cells were treated with pertussis toxin by addition of toxin to the culture media at a final concentration of 100 or 200 ng/ml. Control cells were seeded from the same stock of cells into identical vessels and batches of media not supplemented with pertussis toxin. After 10-h treatment, the cell lysates were resolved on SDS-PAGE, transferred to Hybond ECL nitrocellulose membrane, and analyzed by immunoblotting with anti-NTE or anti-Gβ2 antibodies.

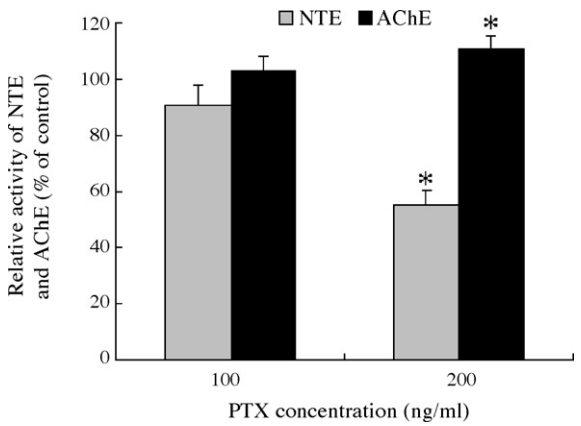

Fig. 5. Effect of pertussis toxin on the activity of NTE and AChE in SH-SY5Y cells. SH-SY5Y cells were treated with pertussis toxin by addition of toxin to the culture media at a final concentration of 0, 100, or 200 ng/ml. The activities of NTE and AChE were assayed. The basal activity of NTE and AChE in control cells was 15.92 ± 1.39 nmol phenol/min/mg protein and 20.15 ± 0.26 nmol acetylthiocholine hydrolyzed/min/mg protein, respectively. Data are presented as a percentage of the activity in control cells. Results are mean ± S.E. (bars) from three independent tests. Statistical analysis of control cells vs. pertussis toxin-treated cells was carried out by ANOVA. $^{*}p < 0.05$, compared with controls, $n = 3$.

PTX-catalyzed ADP-ribosylation of G alpha-subunits, attenuation of the inhibitory regulation of adenylate cyclase, and attenuation of the ability of GTP to induce an agonist-specific shift in receptor affinity; the second group of late effects included the decline in G beta-subunit levels and the progressive enhancement of the stimulatory pathway of adenylate cyclase (Watkins, Northup, & Malbon, 1989). So, the effect of longer PTX treatment on the activity of NTE was investigated. The results indicated that 10-h PTX treatment did not affect the cell viability (data not shown). The expression of NTE protein was not changed by PTX treatment, but Gβ2 was inhibited in a dose-dependent manner (Fig. 4). As shown in Fig. 5, NTE activity in the cells treated with 200 ng/ml PTX was reduced to 55% compared to the control cells, while the activity in cells treated with 100 ng/ml PTX was decreased a little. In contrast, there was less than 10% increase of AChE activity in PTX-treated cells compared with that in control cells. These data further suggested that the decline in G beta-subunit levels resulted in the decrease of NTE activity with no change in protein expression.

4. Discussion

NTE is a novel phospholipase B protein that is highly conserved among species including insects, nematodes, yeast, and bacteria, and plays a role in phosphatidylcholine homeostasis (Lush et al., 1998; Muhlig-Versen et al., 2005; Zaccheo et al., 2004). The deduced primary sequence of NTE (1327 amino acids) revealed that NTE comprised an amino-terminal regulatory domain and a carboxyl-terminal catalytic domain (Lush et al., 1998). The amino-terminal regulatory domain included three speculated cAMP-binding domain, the work of the possible direct activation of NTE by cAMP is under continuing investigation in another laboratory (Glynn, 2005). However, we have shown here that NTE can interact with protein Gβ2 (or specific Gβγ complex) through its catalytic domain, and that depletion of Gβ2 subunit by RNAi or drugs can lead to down-regulation of the activity of NTE.

The heterotrimeric guanine nucleotide-binding proteins (G proteins) is composed of three subunits, Gα, Gβ, and Gγ, each of which is also composed of many isoforms (Cabrera-Vera et al., 2003; Hamm, 1998). The heterotrimeric complex is attached to the cytoplasmic surface of the plasma membrane through the prenylated Gγ (Clapham & Neer, 1997). This heterotrimeric complex is inactive in the GDP-bound state. Once ligands bind to their G protein-coupled receptors, GDP on Gα is catalyzed to GTP, leading to dissociation of the GTP-bound Gα from the Gβ/Gγ heterodimer (Hamm, 1998). Liberated GTP-Gα and the Gβ/Gγ complex then interact with their downstream effector molecules and exert their biological actions. In recent years, the βγ complex has received more attention and appears to play a significant role in the G-protein-signaling pathway. Usually Gβ in the βγ complex exerts the basis of specificity of effector modulation. Different βγ complexes do not have equiva-

lent abilities to couple a subunit to receptors or to regulate effectors (Yan & Gautam, 1997). This means multiple β and γ isoforms are required to accommodate the diverse signaling pathways in biological evolution (Rosskopf et al., 2003). Herein, we have only found Gβ2 and its variant Gβ2-like I interacted with the C-terminal domain of NTE in yeast two-hybrid experiment. This indicates the specificity of interaction between these proteins.

Gβ contains a portion characterized as a seven times–repeated blade-like structure, called a WD repeat (Clapham & Neer, 1997; Smith, Gaitatzes, Saxena, & Neer, 1999). Crystallographic analyses revealed that all WD-repeats of Gβ are made up of four twisted strands and are arranged in a ring, thus, forming a propeller-like structure (Clapham & Neer, 1997). Gβ2 is widely expressed throughout the brain (Betty, Harnish, Rhodes, & Cockett, 1998). It has been shown that the WD repeat is a new interaction motif, through which forms the scaffold for multimeric protein complexes with activators and effectors bound together by adaptors in a precise spatial arrangement to ensure proper function (Dell et al., 2002). The Gβ2/Gγ complex binds to and modulates the activity of diverse molecules: several forms of potassium and calcium ion channels, glucocorticoid receptor and ataxin 10 (Kino et al., 2005; Li et al., 2005; Waragai et al., 2006). In this paper, the interaction of Gβ2 with NTE was confirmed by coimmunoprecipitation assay. Components of the G protein system are presumably strictly located at the inner surface of the plasma membrane attached to it through the prenylated Gγ (Clapham & Neer, 1997). However, the localization of Gβ2 is distributed in the cytoplasm and the nucleus, as well as at the cytoplasmic surface of the plasma membrane (Kino et al., 2005). Previous experiments showed that NTE molecule is exposed on the cytoplasmic face of ER membranes and its activity requires membrane association, which requires the TM, regulator, and catalytic domains (Li et al., 2003). The membrane scaffold complex plays a role in maintaining the activity of NTE. This effect is easily disrupted by addition of detergent and cannot be rescued through simple addition of the NEST protein and/or phospholipids alone (Atkins, Luthjens, Hom, & Glynn, 2002). Gβ2 can migrate between subcellular compartments, such as between the cytoplasm and the nucleus or the plasma membrane, depending on the type of stimuli and the responsive signaling molecules activating the cell (Kino et al., 2005). Therefore, NTE activity may be changed by stimulation through Gβ2 translocation.

Previous study showed that the chemotactic response to the complement factors C5a and C3a is ablated in cells that the expression of Gβ2 subunit was silenced by lentiviral delivery of small interfering RNA (Hwang, Fraser, Choi, Qin, & Simon, 2004). Gβ2 transient antisense experiment showed that down-expression of Gβ2 can lead to inhibition of cell proliferation with cell death after a 4-day treatment and that this protein may play a potential role in the regulation of cell proliferation and microtubule and mitotic spindle organization in mammalian cells (Wu, Huang, Chiu, & Lin, 2001). The same fragile behavior has been found in our subcloned siGβ2-a3 cell line, which showed different cell morphology, higher apoptosis, required a high serum concentration in the medium to maintain its growth. Moreover, silencing the expression of Gβ2 or treatment with PTX for a longer time down-regulated NTE activity without any change in the protein level, which suggests that NTE and Gβ2 form an active complex at its C-terminal domain.

In summary, Gβ2 subunit interacts directly with NTE and silencing of Gβ2 down-regulates the activity of NTE, which indicates Gβ2 plays a role in maintaining the activity of NTE and suggests there is a novel regulatory mechanism for NTE activity. It will be of interest to further investigate the interaction pattern of the NTE and Gβ2 complex and determine the functionality of this interaction.

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References

Akassoglou, K., Malester, B., Xu, J., Tessarollo, L., Rosenbluth, J., & Chao, M. V. (2004). Brain-specific deletion of neuropathy target esterase/swisscheese results in neurodegeneration. *Proceedings of the National Academy of Sciences USA*, *101*, 5075–5080.

Atkins, J., & Glynn, P. (2000). Membrane association of and critical residues in the catalytic domain of human neuropathy target esterase. *Journal of Biological Chemistry*, *275*, 24477–24483.

Atkins, J., Luthjens, L. H., Hom, M. L., & Glynn, P. (2002). Monomers of the catalytic domain of human neuropathy target esterase are active in the presence of phospholipids. *Biochemical Journal*, *361*, 119–123.

Betty, M., Harnish, S. W., Rhodes, K. J., & Cockett, M. I. (1998). Distribution of heterotrimeric G-protein beta and gamma subunits in the rat brain. *Neuroscience*, *85*, 475–486.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, *72*, 248–254.

Cabrera-Vera, T. M., Vanhauwe, J., Thomas, T. O., Medkova, M., Preininger, A., Mazzoni, M. R., & Hamm, H. E. (2003). Insights into G protein structure, function, and regulation. *Endocrine Reviews*, *24*, 765–781.

Chang, P. A., Chen, R., & Wu, Y. J. (2005). Reduction of neuropathy target esterase does not affect neuronal differentiation, but moderate expression induces neuronal differentiation in human neuroblastoma (SK-N-SH) cell line. *Brain Research Molecular Brain Research*, *141*, 30–38.

Chang, P. A., Wu, Y. J., Chen, R., Li, M., Li, W., & Qin, Q. L. (2005). Inhibition of neuropathy target esterase expressing by antisense RNA does not affect neural differentiation in human neuroblastoma (SK-N-SH) cell line. *Molecular and Cellular Biochemistry*, *272*, 47–54.

Clapham, D. E., & Neer, E. J. (1997). G protein beta gamma subunits. *Annual Review of Pharmacology and Toxicology*, *37*, 167–203.

Dell, E. J., Connor, J., Chen, S., Stebbins, E. G., Skiba, N. P., Mochly-Rosen, D., & Hamm, H. E. (2002). The beta gamma subunit of heterotrimeric G proteins interacts with RACK1 and two other WD repeat proteins. *Journal of Biological Chemistry*, *277*, 49888–49895.

Dremier, S., Kopperud, R., Doskeland, S. O., Dumont, J. E., & Maenhaut, C. (2003). Search for new cyclic AMP-binding proteins. *FEBS Letters*, *546*, 103–107.

Glynn, P. (2005). Neuropathy target esterase and phospholipid deacylation. *Biochimica et Biophysica Acta (BBA)—Molecular and Cell Biology of Lipids*, *1736*, 87–93.

Gorun, V., Proinov, I., Baltesuc, V., Balaban, G., & Barzu, O. (1978). Modified Ellman procedure for assay of cholinesterase in crude enzymatic preparations. *Analytical Biochemistry*, *86*, 324–326.

Hamm, H. E. (1998). The many faces of G protein signaling. *Journal of Biological Chemistry*, *273*, 669–672.

Hwang, J. I., Fraser, I. D., Choi, S., Qin, X. F., & Simon, M. I. (2004). Analysis of C5a-mediated chemotaxis by lentiviral delivery of small interfering RNA. *Proceedings of the National Academy of Sciences USA*, *101*, 488–493.

Johnson, M. K. (1982). The target for initiation of delayed neurotoxicity by organophosphorus esters: Biochemical studies and toxicological applications. *Reviews in Biochemical and Toxicology*, *4*, 141–212.

Kino, T., Tiulpakov, A., Ichijo, T., Chheng, L., Kozasa, T., & Chrousos, G. P. (2005). G protein beta interacts with the glucocorticoid receptor and suppresses its transcriptional activity in the nucleus. *Journal of Cell Biology*, *169*, 885–896.

Li, Y., Dinsdale, D., & Glynn, P. (2003). Protein domains, catalytic activity, and subcellular distribution of neuropathy target esterase in mammalian cells. *Journal of Biological Chemistry*, *278*, 8820–8825.

Li, X., Hummer, A., Han, J., Xie, M., Melnik-Martinez, K., Moreno, R. L., Buck, M., Mark, M. D., & Herlitze, S. (2005). G protein beta2 subunit-derived peptides for inhibition and induction of G protein pathways. Examination of voltage-gated Ca^{2+} and G protein inwardly rectifying K^{+} channels. *Journal of Biological Chemistry*, *280*, 23945–23959.

Lush, M. J., Li, Y., Read, D. J., Willis, A. C., & Glynn, P. (1998). Neuropathy target esterase and a homologous Drosophila neurodegeneration-associated mutant protein contain a novel domain conserved from bacteria to man. *Biochemical Journal*, *332*, 1–4.

Moser, M., Li, Y., Vaupel, K., Kretzschmar, D., Kluge, R., Glynn, P., & Buettner, R. (2004). Placental failure and impaired vasculogenesis result in embryonic lethality for neuropathy target esterase-deficient mice. *Molecular and Cellular Biology*, *24*, 1667–1679.

Muhlig-Versen, M., da Cruz, A. B., Tschape, J. A., Moser, M., Buttner, R., Athenstaedt, K., Glynn, P., & Kretzschmar, D. (2005). Loss of Swiss cheese/neuropathy target esterase activity causes disruption of phosphatidylcholine homeostasis and neuronal and glial death in adult *Drosophila*. *Journal of Neuroscience*, *25*, 2865–2873.

Murray, J. P., & McMaster, C. R. (2005). Nte1p-mediated deacylation of phosphatidylcholine functionally interacts with Sec14p. *Journal of Biological Chemistry*, *280*, 8544–8552.

Quistad, G. B., Barlow, C., Winrow, C. J., Sparks, S. E., & Casida, J. E. (2003). Evidence that mouse brain neuropathy target esterase is a lysophospholipase. *Proceedings of the National Academy of Sciences USA*, *100*, 7983–7987.

Rosskopf, D., Nikula, C., Manthey, I., Joisten, M., Frey, U., Kohnen, S., & Siffert, W. (2003). The human G protein beta4 subunit: Gene structure, expression, G gamma and effector interaction. *FEBS Letters*, *544*, 27–32.

Smith, T. F., Gaitatzes, C., Saxena, K., & Neer, E. J. (1999). The WD repeat: A common architecture for diverse functions. *Trends in Biochemical Sciences*, *24*, 181–185.

Waragai, M., Nagamitsu, S., Xu, W., Li, Y. J., Lin, X., & Ashizawa, T. (2006). Ataxin 10 induces neuritogenesis via interaction with G-protein beta2 subunit. *Journal of Neuroscience Research*, *83*, 1170–1178.

Watkins, D. C., Northup, J. K., & Malbon, C. C. (1989). Pertussis toxin treatment in vivo is associated with a decline in G-protein beta-subunits. *Journal of Biological Chemistry*, *264*, 4186–4194.

Winrow, C. J., Hemming, M. L., Allen, D. M., Quistad, G. B., Casida, J. E., & Barlow, C. (2003). Loss of neuropathy target esterase in mice links organophosphate exposure to hyperactivity. *Nature Genetics*, *33*, 477–485.

Wu, H. C., Huang, P. H., Chiu, C. Y., & Lin, C. T. (2001). G protein beta2 subunit antisense oligonucleotides inhibit cell proliferation and disorganize microtubule and mitotic spindle organization. *Journal of Cellular Biochemistry*, *83*, 136–146.

Yan, K., & Gautam, N. (1997). Structural determinants for interaction with three different effectors on the G protein beta subunit. *Journal of Biological Chemistry*, *272*, 2056–2059.

Zaccheo, O., Dinsdale, D., Meacock, P. A., & Glynn, P. (2004). Neuropathy target esterase and its yeast homologue degrade phosphatidylcholine to glycerophosphocholine in living cells. *Journal of Biological Chemistry*, *279*, 24024–24033.