Membrane-bound Carboxypeptidase E Facilitates the Entry of Eosinophil cationic protein to Neuroendocrine Cell

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Abberviations:

ARF6, ADP-ribosylation factor 6; CPB, carboxypeptidase B; CPD, carboxypeptidase D; CPE, carboxypeptidase E; CPM, carboxypeptidase M; CPN, carboxypeptidase N; ECP, eosinophil cationic protein; EDN, eosinophil derived neurotoxin; eGFP, enhance green fluorescent protein.

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SYNPOSIS

Eosinophil cationic protein (ECP) is a major component of eosinophil granule proteins and is used as a clinical bio-marker for asthma and allergic inflammatory disease. ECP has been implicated in damage to the cell membrane of many tissue types, but the mechanism is not well known. In this study, mECP-eGFP-6H, a recombinant fusion protein containing mature ECP (mECP), green fluorescence protein (eGFP) and Histag, has been expressed, purified and added to GH3 neuroendocrine cells to study the internalization ability of ECP. We found that mECP-eGFP-6H entered into GH3 neuroendocrine cells and inhibited the growth of the cells with the IC_{50} of 0.8 μM. By yeast two-hybrid screening and immunoprecipitation, we have identified a specific protein-protein interaction between mature mECP and carboxypeptidase E (CPE), a well characterized metalloprotease. Further i*n vivo* yeast two-hybrid screening has also revealed that residues 318 to 387 located in a region of unknown function in mature CPE are indispensable for association with mECP. In addition, the uptake of mECP-eGFP-6H is suppressed by dominant-negative expression of the recycling defect mutant preproHA-$CPE_{S471A,E472A}$ in GH3 cells, suggesting that the entrance of mECP-eGFP-6H is associated with the recycling of CPE in GH3 cells. Taken together, we have demonstrated that CPE possesses a novel function to facilitate the entry of ECP to neuroendocrine cell, and such endocytosis process allows the cytotoxic ECP to inhibit growth of the target cells.

INTRODUCTION

Eosinophil cationic protein (ECP) is one of the major components of the eosinophilic granules and has a molecular mass ranging from 16 to 21.4 kDa due to varying degrees of glycosylation. It is classified as a member of the ribonuclease A (RNase A) superfamily and exhibits differential biological effects both *in vivo* and *in vitro* [1]. Among eight human RNases, ECP is most homologous to eosinophil derived neurotoxin (EDN). ECP is bactericidal [2] and helminthotoxic [3] [4], and is cytotoxic to tracheal epithelium [5, 6]. When intrathecal injected into rabbits, ECP or EDN elicit the Gordon phenomenon, a syndrome manifested by ataxia, muscular rigidity, paralysis, and tremor that may lead to death, and most of the time the characteristic Purkinje cell toxicity occurs [7, 8]. Although the mechanism of its cytotoxicity is not completely understood, it is suggested that the pore-forming activity of ECP, but not the ribonuclease activity, plays a key role in destabilizing lipid membranes of the target cells [9, 10]. This is consistent with data showing that the cytotoxicity of ECP is greater than that of EDN [11].

It has been reported that ECP exhibits growth-inhibitory effects on several cell types [12, 13]. Despite the high degree of sequence homology between ECP and other members in the RNase A superfamily, ECP exhibits weak but definite ribonuclease activity. ECP is able to effectively internalize the cytosol and escape from proteolytic attack. It is accumulated in the cytosol to large excess over the concentration of RNase inhibitor such that degradation of cytosolic RNA molecules occurs [13]. The cellular internalization of proteins often occurs via a specific energy-dependent endocytosis pathway, through either clathrin-coated or non-clathrin-coated endosomes. Maeda, *et. al.* suggested that ECP may interact preferentially with a receptor or binding protein on the cell surface [12]. Hence,

ECP appears to enter cells via energy-dependent endocytosis. However, no ECP-interacting protein involved in this endocytosis process has ever been identified.

In this study, we have assessed the effect of mECP-eGFP-6H fusion protein on the growth of the mammalian neuroendocrine cell line, GH3. The results show that mECP-eGFP-6H enters the cell whereas RNase A does not. In addition, mECP-eGFP-6H inhibits growth of GH3 cells with IC_{50} of 0.8 μM. Using mECP as a bait to investigate its interacting proteins *via* yeast two-hybrid system, we found that ECP interacted with the transmembrane, lipid raft-associated prohormone sorting receptor, carboxypeptidase E (CPE). Blocking the maturation of CPE-associated secretory granules and the dominant-negative expression of $CPE_{S473A,E474A}$, a recycling defect mutant in GH3 cell line both interfered with the internalization of mECP-eGFP-6H, indicating that interaction between mECP and CPE plays a major role in internalization of mECP to the neuroendocrine cells. Our results provide the first demonstration of a non-surface receptor mediated mechanism responsible for the entrance of cytotoxic RNase into the target cells.

EXPERIMENTAL

Cell culture and transfection

GH3 cells were obtained from the American Type Culture Collection (Manassas, VA) and cultured in F12K (Sigma) containing 15% horse serum and 2.5% fetal bovine serum (FBS), penicillin/streptomycin and incubated at 37 ^{o}C, 5% CO_2. Transient transfections were performed using TransFast^{TM} (Promega) according to the manufacturer's protocol. In brief, GH3 cells were subcultured one day before transfection. Subcultured GH3 cells were harvested by trypsin/EDTA treatment, and collected by centrifugation at 500 *g*, and then washed twice with PBS. Finally, the cells were resuspended in serum-free F12K medium and the cell number was counted using the dye-exclusion hemocytometer procedure with 0.4% trypan blue in HBSS. 2×10^6 GH3 cells were transfected with 2 μg DNA, and incubated at 37 ^{o}C, 5% CO_2 for 48 hr to express the target protein.

Preparation of recombinant mECP-eGFP-6H and eGFP-6H fusion proteins

Human ECP cDNA was isolated and expressed in an *Escherichia coli* T7 expression system by modified method as described previously [14]. The *egfp* gene was cloned into pET23b(+) vector between the *Hind*III and *Xho*I sites to generate *egfp*-pET23b(+). The *mecp* gene was subsequently introduced between the *Nde*I and *Hind*III sites to generate the *mecp-egfp*-pET23b(+). Both recombinant DNAs were transformed into *Escherichia coli* BL21(DE3) for protein expression. One ml of overnight culture of each transformant were inoculated in 1 liter of LB containing 200 μg/ml carbenicillin (Sigma), and grown overnight at 20 ^{o}C with shaking. Isopropyl-1-thio-β-D-galactopyranoside (IPTG) (Sigma) was added to a final concentration of 2 mM, and the bacteria were harvested 48 hr after induction.

Recombinant mECP-eGFP-6H and eGFP-6H proteins in the soluble portion of bacteria cell lysates were subject to purification employing His-Bind@ affinity column Chromatography (Novagen).

MTT assay for GH3 cell growth

The effect of mECP-eGFP-6H on the growth of GH3 cell line was assessed by colorimetric assay using 3-(4,5-dimethylthiazol-2-yl)-diphenyl-tetrazolium bromide (MTT) by modified method as previously described [12]. Cells were plated into 96-well plates in F12K media containing 15% horse serum and 5% fetal bovine serum at 500 cells per well for 24 hr prior to addition of mECP-eGFP-6H at the indicated concentration (0-2.5 μM). Three days after plating, the medium was replaced with fresh medium containing mECP-eGFP-6H at the same concentration. After a further three days of cultivation, MTT (5 mg/ml in PBS) was added, and cell growth was monitored by measuring A_{570}.

Uptake of mECP-eGFP6H into GH3 cells

GH3 cells were cultured as described previously. 0.5 μM of mECP-eGFP6H was directly added to the culture medium in the presence and absence of 15 mM NH_4Cl or 5 μM forskolin and incubated for different durations. Cells were collected by centrifugation at 3000 rpm and washed twice with PBS, then treated with cell-lysis buffer for further analysis. For fluorescence microscopy experiments, bovine RNase A (Sigma) was labeled with rhodamine B isothiocyanate (RITC; Sigma) as previously described [15]. GH3 cells were seeded into a six-well plate and grown on coverslips at 40000 cells per well for 24 hr. mECP-eGFP-6H, eGFP-6H or RITC labeled RNase A was separately added to the culture medium to a final concentration of 0.5 μM and incubated for 15 min to 3 hr. After incubation, cells were washed twice with

ice-cold PBS, fixed with 4% formaldehyde solution at room temperature for 15 min, and the nuclei were stained with 300 nM 4',6-diamidino-2-phenylindole (DAPI, Mloecular Probes), prior to imaging by fluorescence microscopy.

Yeast two-hybrid assay

Yeast two-hybrid screening was performed by using the MATCHMAKER Two-Hybrid System 3 (Clontech) according to manufactor's instructions. For bait vector construction, mature human ECP gene (*mecp*) was amplified by PCR with CATATGGTTCCAAAACTG as the 5'-primer and GAATTCTTAGATGGTGGTATC as the 3'-primer. The PCR fragments were cloned into the *Nde* I and *Eco*RI sites of pGBKT7 (Clontech). The human brain cDNA library (Clontech) was screened for mECP-associated proteins. mECP-bait construct alone did not show autonomous transcriptional activation in the AH109 yeast strain. Thus yeast cells were co-transformed with 0.1 mg cDNA library plasmids and 0.2 mg mECP-bait plasmids according to a basic lithium acetate protocol. The transformants were grown on SD-Ade-His-Leu-Trp plates to select for protein-protein interactions. The β-galactosidase assay was further performed to test *LacZ* reporter gene expression of the clones selected. The library plasmids were then isolated from the positive selections and retransformed into yeast AH109 together with the mECP-bait plasmid for β-galactosidase activity assay. The *ade2*+/*his3*+/*lacZ*+ library plasmids containing the candidate genes were transformed into *E. coli* Top10F' cells and the plasmid DNAs were prepared for sequencing. For quantitative and qualitative assay for specific interaction between mECP and the candidate prey on the AD were co-transformed into yeast host AH109. Clones containing different candidates were scraped and cultured on SD-Ade-His-Leu-Trp plates for qualitative assay of protein-protein interactions. For liquid culture β-galactosidase assay, single colony

was cultured in SD-Leu-Trp medium until OD_{600} reached 0.6. The assay was performed in Z buffer (60 mM Na_2HPO_4; 40 mM NaH_2PO_4, pH 7.0; 10 mM KCl; 1 μM $MgSO_4$); 0.64 mg/ml ONPG (o-nitrophenyl β-D-galactopyranoside, Sigma) was used as substrate and the OD_{420} value of samples after 400 min incubation at 30 ^oC were measured.

Constructions of CPE deletion mutants

To identify the region of CPE for interaction with mECP, various deletions were constructed. The candidate gene $CPE_{154-476}$–pACT2 were sub-cloned into AD vector (pGADT7), and seven serial *C*-terminal deletions of CPE were generated by a standard nested deletion method. Briefly, two restriction enzyme sites, *Bam*HI and *Sac*I, were introduced at the 3'-end of $CPE_{154-476}$ DNA fragment on $CPE_{154-476}$–pGADT7 clone to generate $CPE_{154-476,BamHI,SacI}$–pGADT7. $CPE_{154-476,BamHI,SacI}$–pGADT7 was double digested by *Bam*HI and *Sac*I restriction enzymes, then treated with *Exo*III nuclease at 25 ^oC and samples taked at 30 sec intervals. After *Exo*III digestion, DNAs were treated with S1 nuclease and Klenow enzyme for 15 min at 37 ^oC. Finally, the treated DNA products were ligated by T4 DNA ligase at room temperature for 1 hr then the self-ligated DNAs were transformed into Top10F' for screening.

Immunoprecipitation and Western blot analysis

Equal amounts (1 μg) of either purified eGFP-6H or mECP-eGFP-6H fusion protein were added to the binding buffer (PBS containing 5 mM EDTA and 0.5% NP-40) containing 100 μg GH3 cell lysates and incubated at 4 ^oC overnight. Subsequently, eGFP-6H and mECP-eGFP-6H were subjected to immunoprecipitation from the mixture by adding 1 μg of polyclonal anti-eGFP antibody (Clontech). Protein

A-agarose beads (Amersham Biosciences) were added to the cell lysate and then incubated at 4 ^{o}C overnight on a rotating apparatus. The beads were collected by centrifugation at 6000 rpm and washed twice with PBS. Finally, the pellet was resuspended in 1 X SDS-PAGE sample buffer for further analysis. Monoclonal CPE antibody (CashmereBiotech, Taiwan) and monoclonal ECP109 antibody (BCRC 69019) were used in Western blotting.

Construction of preproHA-CPE$_{S471A,E472A}$ mutation

Human preproHA-CPE$_{S471A,E472A}$ point mutation was made by two-stage PCR method. First, 5'-GAATTCATGGCCGGGCGCGGACGG and 5'-AGCGTAATCTGGTACGTCGTACGCCGGCGCCGCCGCATGC were used to amplify the preproHA fragment. Subsequently, 5'-TACGACGTACCAGATTACGCTCTGCAGCAAGAGGACGGC and 5'-CTCGAGTTAAAAATTTAAAGT*TGCTGC*CATCATTTTCC were used to amplify the HA-CPE$_{S471A,E472A}$ fragment. The HA taq epitope region is underlined and S471A,E472A point mutations are displayed in Italics. Finally, the preproHA and HA-CPE$_{S471A,E472A}$ fragments were used as PCR template to generate the preproHA-CPE$_{S471A,E472A}$. The final PCR fragment was cloned between the *Eco*RI and *Xho*I sites of pcDNA3 (Invitrogen). The sequence of the plasmid cDNA was verified by sequencing analysis.

RESULTS

Preparation of Recombinant mECP-eGFP-6H and eGFP-6H

Mature ECP was fused with eGFP, expressed as mECP-eGFP-6H fusion protein in *E. coli* cells, and the soluble portion was purified by His-Bind$^{@}$ Resin (Novagen). The molecular weight of mECP-eGFP-6H is determined to be 42 kDa. The 27 kDa recombinant eGFP-6H was obtained in a similar manner (figure 1A). The RNase activity of purified recombinant mECP-eGFP-6H was assessed with yeast RNA by the perchloric and precipitation method [16]. It was determined to be about 100 times lower than RNase A.

mECP-eGFP-6H inhibits the growth of neuroendocrine cells

In the presence of 2.5 μM mECP-eGFP-6H, the growth of GH3 cells was completely suppressed, whereas no inhibitory effect was observed upon treatment with either RNase A or eGFP-6H (figure 1B). The result clearly indicated that only the recombinant mECP-eGFP-6H could suppress the growth of GH3 cells. Neither eGFP-6H nor RNase A was able to interfere with the growth of GH3 cells. To monitor the internalization of the RNases, the GH3 cells were incubated with RITC-labeled RNase A or mECP-eGFP-6H at 37 ^{o}C for 3 hr. No RITC-labeled RNase A was detected in the GH3 cells after 3 hr incubation, suggesting that RNase A could not get internalized (figure 2A). However, GH3 cells exhibited increased levels of fluorescence labeling from mECP-eGFP-6H, and most likely located in the cytoplasm rather than the nuclei (figure 2B). This result indicated that only the recombinant mECP-eGFP-6H could enter the GH3 cells. The cell lysates collected from reactions treated with mECP-eGFP-6H for 0, 1 or 2 hr were further analyzed by SDS-PAGE and Western bloting. Figure 2C revealed that the relative amount of

mECP-eGFP-6H taken up by GH3 cells after 2 hr treatment was approximately 1.7 fold higher than that of 1 hr treatment. The increasing amount of mECP-eGFP-6H acumulated in GH3 cells is consistent with inhibition of cell growth.

Secretagogues and lysosomotrophic agents affect mECP-eGFP-6H uptake into cells

To further examine whether the entrance of mECP-eGFP-6H to GH3 cells is associated with the endocytosis and regulated secretion pathway, the cells were pre-treated with secretagogue (5 μM forskolin) or lysosomotrophic (15 mM NH_4Cl) agents in the culture medium for 1 hr prior to addition of 0.5 μM mECP-eGFP-6H fusion protein. Figure 3A shows that the relative amount of mECP-eGFP-6H in GH3 cells was decreased upon treatment with NH_4Cl, presumably due to blockage of the endosomal acidification and the secretion of vesicles. One the other hand, pre-treatment with forskolin resulted in a 2- to 3-fold increase of mECP-eGFP-6H accumulation as shown in figure 3B. These results indicate that the acidic vesicles are involved in the internalization of mECP-eGFP-6H.

CPE interacts with mature ECP in yeast cells

To investigate which cellular proteins interact with mECP, a yeast two-hybrid screening system was constructed consisting of a human brain cDNA library and human ECP as bait. This screen identified one clone containing the last 363 amino acids of human carboxypeptidase E (CPEc). The interaction was tested against ECP and human RNase A (used as a negative control) in a growth assay. In the absence of adenine and histidine, only the yeast expressing ECP could grow under such stringent conditions. The full-length mature human CPE (mCPE) was further cloned to examine its ability to interact with ECP. Figure 4A shows mCPE binds to mECP with a similar affinity as CPEc does, indicating that the *N*-terminal 114 amino acids of

CPE do not affect its interaction with mECP. The expression of mECP-BD and CPE-AD fusion proteins in yeast cells were further detected by Western blotting with anti c-myc and anti-HA antibody, respectively. The molecular weight of mECP-BD, mCPE-AD and CPEc-AD fusion proteins expressed in yeast cells was estimated to be 34 kDa, 65 kDa and 53 kDa, respectively (figure 4B).

mECP is associated with CPE in vitro

Direct interaction between CPE and ECP was further confirmed by immunoprecipitation using purified mECP-eGFP-6H fusion protein and GH3 cell lysates containing endogenous CPE. Equal amounts (1 μg) of either purified eGFP-6H or mECP-eGFP-6H fusion protein were added to the binding buffer containing 1 mg GH3 cell lysates and incubated at 4 ^{o}C overnight. Subsequently, eGFP-6H and mECP-eGFP-6H were subjected to immunoprecipitation from the mixture by a polyclonal anti-eGFP antibody. The immunocomplexes were subjected to Western blot analysis with anti-CPE antibody specifically recognized the *N*-terminal region of CPE. No CPE protein was detected in the former experiments (figure 5, lane 1), whereas a 50 kDa CPE protein was specically detected in the latter experiment (figure 5, lane 2). These results provide additional evidence of physical interaction between mECP and CPE *in vitro*.

The region from 318 to 387 of mature CPE is indispensable for association with mECP in yeast

To figure out which region in mCPE is responsible for the interaction with mECP, seven deletion mutants of CPEc were constructed by the nested deletion method (figure 6A) and the binding of mECP to these truncated CPE mutants were examined in yeast cells. The reporter gene expression assays and cell growth results indicated

that the *C*-terminal truncated forms of CPEc-nested-5 (amino acids 114–318), CPEc-nested-6 (amino acids 114–217) and CPEc-nested-7 (amino acids 114–156) could not interact with mECP in yeast cells. However, the reporter gene expression was activated by the CPEc-nested 4 (amino acids 114–387) containing a domain (amino acids 318–387) of unknown function. β-galactosidase activity assay was used to monitor *Gal 4* reporter gene expression, and it was found that no activity difference existed among the nested deletion mutants 1 to 4, whereas mutant 5 to 7 all lost the enzymatic activity (Fig 6B, right). Primary sequence alignment among human CPE and its duck homolog protein, CPD domain II, as well as two human homolog proteins, carboxypeptidase M (CPM) and carboxypeptidase N (CPN), reveals that the mECP-associating region containing residues 318 to 387 of mCPE is highly conserved (figure 6C).

The uptake of mECP-eGFP-6H fusion protein is blocked by dominant-negative expression of the preproHA-CPE$_{S471A,E472A}$ in GH3 cells

To further examine the effect of the uptake of mECP-eGFP-6H, GH3 cells were transiently transfected with preproHA-CPE$_{S471A,E472A}$-pcDNA3 or pcDNA3 vector, and incubated to overexpress the recombinant protein. After 48 hr incubation, both clones were incubated with 0.5 μM mECP-eGFP-6H for an additional 1 to 3 hr. The expression of the recycling defect protein preproHA-CPE$_{S471A,E472A}$ was further examined by SDS-PAGE and Western blotting using anti-HA antibody (figure 7A). The results demonstrated that the dominant negative preproHA-CPE$_{S471A,E472A}$ was successfully expressed in GH3 cells and significantly decreased the uptake of mECP-eGFP-6H fusion protein into the GH3 cells. One the other hand, not much difference in the internalization of mECP-eGFP-6H could be observed in the cells transfected with pcDNA3 only (figure 7B).

DISCUSSION

Our result shows that mECP not only gets internalized into GH3 neuroendocrine cells but also inhibits the growth of the cells. This phenomenon is consistent with previously reported growth-inhibitory effects of ECP to mammalian leukemia, epidermoid carcinoma and breast carcinoma cell lines [12, 13]. We found that the growth of GH3 cells was completely inhibited by mECP-eGFP-6H at a concentration of 2.5 μM and the IC_{50} is 0.8 μM. Both values are lower than those for other cell lines used in previous studies under similar condition, indicating that neuroendocrine cells are particularly sensitive to mECP.

The entrance of mECP-eGFP-6H into the GH3 cells has been shown to be associated with the secretion cycle of the regulated secretory granules, indicating that mECP may interact with the component proteins within the secretory granules. Yeast two-hybrid library screening and immunoprecipitation experiments provide two lines of evidence that mECP specifically interacts with CPE. CPE was first purified from bovine brain and is also called enkephalin convertase or CPH. It is a CPB-like metalloenzyme associated with the biosynthesis of many peptide neurotransmitters and hormones. The role of CPE in the removal of carboxyl-terminal basic residues exposed by the endoproteases is known to be necessary for the processing of a large number of the protein precursors [17]. Previous studies suggested that CPE expressed in breast cancer and small-cell carcinoma of the lung cells was necessary to process neuropeptides for autocrine loops [18, 19]. To further investigate the critical role that CPE plays in different cells, in terms of facilitating internalization of recombinant ECP, we have examined the expression of CPE in mammalian cell lines such as K562 and A431 by Western blot analysis. The results clearly reveal that CPE does express at different level in these cell lines (data not shown), meaning that

expression of CPE in these cells may be associated with the ECP-uptake effect. Several metallocarboxypeptidases have now been demonstrated to be multi-functional. For example, duck gp180 which has carboxypeptidase D activity binds to preS envelope protein of duck hepatitis B virus particles [20] and human angiotensin-converting enzyme 2 (ACE2) is a receptor for SARS coronavirus [21]. The present study indicates that membrane-bound CPE facilitates the entry of recombinant ECP to cells. To our knowledge, this is the first report showing non-surface receptor protein is directly responsible for the internalization of recombinant mECP.

Further analysis of CPE deletion clones employing yeast two-hybrid system has revealed that residues 318 to 387 in mature CPE are indispensable for association with mECP. This region is highly conserved in all regulatory carboxypeptidase members and located in a domain of previously unknown function. Our results provide the first evidence for the biological function of this particular domain. Both CPE and CPD are major peptide processing enzymes present in the secretory pathway of neuroendocrine cells and can recycle from the cell surface. However, they are enriched in the different parts of the pathway [22]. The subcellular localization of the former is in the regulated secretory vesicles but the latter is in the *trans*-Golgi network [22, 23]. The mECP-interacting region on CPE is highly conserved with the second domain of CPD, indicating that specific protein-protein interaction between mECP and CPE may also occur between mECP and CPD. Since CPD is a carboxypeptidase widely distributed in different tissues, and not only limited to neuroendocrine tissues, such an interaction may contribute to the broad role for CPD in processing proteins that transit the secretory pathway.

RNases constitute a large superfamily spanning many species. In addition to simple ribonuclease activity, some members of the RNase A superfamily have

cytotoxic activity toward tumor cell lines. For example, bovine seminal (BS) RNase, onconase, human eosinophil derived neurotoxin with four extra residues SLHV at *N*-terminus, and hECP all showed cytotoxicity to different mammalian cell lines [24-26]. hECP is one member of human RNase A family, and the growth suppression effect of several types of mammalian cell lines have been well studied. The growth-inhibitory effect of ECP is cytostatic but not cytotoxic, mainly due to its extraordinary stability among human RNases. Previous studies suggest that onconase, angiogenin and BS-RNase can bind to the specific receptor-like-site in the plasma membrane, but no specific receptor protein has ever been identified yet [27]. In addition, the internalization mechanism of α-sarcin, onconase and toxic G88R RNase A has been demonstrated to be clathrin independent and dynamin independent [28, 29]. Hence, researchers have suggested that ECP may be located in the vesicles or endosomes, and that there are cell surface receptors for ECP [12, 13].

We have demonstrated for the first time that hECP interacts with CPE, a metallocarboxypeptidase wildly distributed in central nervous system. Previous studies have demonstrated that the *C*-terminal 25 amino acids of CPE are critical for the membrane binding and recycling of CPE [30, 31]. The S471A and E472A point mutations on CPE block the interaction between CPE and ARF6 and abolish the recycling of CPE from plasma membrane to the *trans*-Golgi network in a clathrin-independent manner [31]. Although the primary structure of CPE shows high identity with the second domain of CPD, our result shows that the uptake of mECP-eGFP-6H is suppressed by dominant-negative expression of the preproHA-$CPE_{S471A,E472A}$ recycling defect mutant in GH3 cells, suggesting that uptake of mECP-eGFP-6H is mainly associated with the recycling of CPE in GH3 cells. Therefore, it is expected that cells containing sufficient amount of CPE can be potential target cells of mECP.

In the present study, we have demonstrated that specific interaction between mECP and CPE, the recycling lipid raft-associated prohormone processing enzyme, plays a critical role in the entrance of mECP to the neuroendocrine cells and causes the cell growth inhibitory effect of the cells. The entrance of mECP is associated with the recycling of CPE, and is consistent with the hypothesis that extracellular RNases uptake is via a clathrin-independent endocytosis pathway. Furthermore, this special cell entry mechanism through CPE may provide clues for the loss of Purkinje cells in the cerebellum of Gordon phenomenon. Although CPE is identified to be a key player in the molecular mechanism of internalization and cytostatic effect of mECP, it still requires further investigation to search for other component proteins responsible for different cytotoxic RNases.

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FIGURE LEGEND

Figure 1. Suppression of GH3 cell growth in the presence of mECP-eGFP-6H.

(A) The molecular weight of mECP-eGFP-6H fusion protein (lane 1) and eGFP-6H (lane 2) are about 42 kDa and 27 kDa respectively. (B) Growth of GH3 cells was monitored by MTT assay, the percentages of viable cells under various concentration of RNase A (●), eGFP-6H (○), and mECP-eGFP-6H (▼) were plotted. Each experiment was carried out in triplicate and standard derviation was calculated and depicted in each vertical line.

Figure 2. GH3 cells treated with RITC-labeled mECP-eGFP-6H and RITC-labeled RNase A.

GH3 cells were seeded into the six-well plates and grown on coverslips at 40000 cells per well. After 24 hr, 0.5 μM mECP-eGFP-6H and RITC-labeled RNase A were added to the culture medium respectively, and the cells were fixed and detected by fluorescence microscopy at the time indicated and nuclei of the cells were stained with DAPI (blue). (A) GH3 cells treated with RITC-labeled RNase A for 3 hr. No RITC-labeled RNase A was detected. (B) GH3 cells treated with mECP-eGFP-6H for 0 hr and 3 hr. mECP-eGFP-6H was visualized (green). (C) Relative amount of mECP-eGFP-6H in GH3 cells at different incubation time were detected by Western blot employing monoclonal anti-ECP109 antibody.

Figure 3. Effects on mECP-eGFP-6H uptake into GH3 cells by lysosomotrpphic and secretagogues agent.

Lanes 1, 2, 3 in A and B were GH3 cells treated with medium containing 0.5 μM mECP-eGFP-6H for 1 hr, 2 hr, and 3 hr, respectly. (A) GH3 cells were pre-treated

with 15 mM NH_4Cl within culture medium 1 hr at 37°C, 5% CO_2, and then treated 0.5 μM mECP-eGFP-6H fusion protein for 1 hr (lane 4), 2 hr (lane 5) and 3 hr (lane 6). (B) GH3 cells were pre-treated with 5 μM forskolin within culture medium 1 hr at 37°C, 5% CO_2, and then treated 0.5 μM mECP-eGFP-6H fusion protein for 1 hr (lane 4), 2 hr (lane 5), and 3 hr (lane 6). The uptake of mECP-eGFP-6H was detected by Western blotting using anti-ECP109 monoclonal antibody.

Figure 4. Yeast two-hybrid screen using mECP as bait and a human brain cNDA library identified a clone containing the last 363 amino acids of CPE.

mECP was cloned into bait vector pGBKT7. Yeast strain AH109 was co-transformed with mCPE-pGADT7 or the pGADT7 library clone containing the *C*-terminal 363 amino acids of CPE (CPEc-pGAD7). (A) Transformants were spread onto SD-Ade-His-Leu-Trp plate. Vigorous growth was observed for cells co-expressing the mECP bait plasmid and two CPE clones at the high-strengthy selection plate. (1) mCPE, (2) CPEc. (B) The expression of mECP-BD and CPE-AD fusion proteins in yeast cells were detected by Western blotting with anti c-myc and anti-HA antibody, respectly. Mature CPE-AD (top, lane 1), CPEc-AD (top, lane 2) and mECP-BD (bottom, lane 1 and 2) fusion proteins were expressed in yeast cells.

Figure 5. ECP is associated with CPE *in vitro*.

The direct interaction between CPE and ECP was further confirmed by immunoprecipitation using purified mECP-eGFP-6H fusion protein and GH3 cell lysates with monoclonal anti-CPE antibody (Lanes 1 to 3) or anti-6His antibody (Lanes 4 to 7). The cell lysates were collected and 100 μg lysate proteins were pre-mixed with 1 μg of eGFP-6H (lane 1) or 1 μg of mECP-eGFP-6H fusion protein (lane 2) in IP buffer. Lane 3 is GH3 cell lysate loading control.

Immunoprecipitation was performed using a rabbit polyclonal anti-eGFP antibody and protein A-Sepharose, subsequently SDS-PAGE and transfering to PVDF membrane were performed. Lanes 4 and 5: IP efficiency control of mECP-eGFP-6H or eGFP-6H with anti-eGFP antibody, respectively. Lanes 6 and 7: the input protein of mECP-eGFP-6H or eGFP-6H.

Figure 6. Investigation of the interacting region on CPE.

(A) CPEc-pGADT7 was used as template to generate seven nested deletion mutants (left). The numbers at both ends of each fragment represent the positions of the terminal amino acid residues in the CPE deletion mutants. Interactions were indicated by growth (+) or no growth (-) on SD-Ade-His-Leu-Trp plate (right). (B) The expression of AD-CPEc and AD-nested CPE deletion mutant fusion proteins was detected by Western blotting using anti-HA antibody (top left). Lanes 1 and 9: AD-CPEc, lanes 2 to 8: nested 1 to 7, respectively. The expression of BD-mECP (lanes 1 to 8) or BD-hRNase A (lane 9) fusion proteins was detected by western blotting with anti c-myc antibody (bottom left). The interacting strength between mECP and CPEc nested deletion mutants were monitored with the *Gal 4* reporter gene expression by β-galactosidase activity assay (right). (C) Amino acid sequence alignment of human CPE 318 to 387 with duck CPD domain II (CPD D2, 1QMU), human CPM (EC 3.4.17.12) and human CPN (EC 3.4.17.3).

Figure 7. Internalization of mECP-eGFP-6H fusion protein is prevented by expression of the preproHA-CPE$_{S471A,E472A}$ cytoplasmic tail mutation protein in GH3 cells.

(A) pcDNA3 vector (lane 1) and preproHA-CPE$_{S471A,E472A}$-pcDNA3 (lane 2) were transfect into GH3 cells relatively The expression of recombinant protein was

analyzed by SDS-PAGE (top) or western blotting using monoclonal anti-CPE antibody (A, bottom). (B) GH3 cells transfected with pcDNA3 or preproHA-$CPE_{S471A,E472A}$-pcDNA3 (CPE_{DN}-pcDNA3) were incubated with 0.5 μM mECP-eGFP-6H for indicated time. The mECP-eGFP-6H fusion protein in GH3 cells were detected by western blotting employing anti-ECP109 monoclonal antibody (top). Relative amount of mECP-eGFP-6H was quantitatively analyzed with α tubulin as an internal control (bottom).

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Figure 1

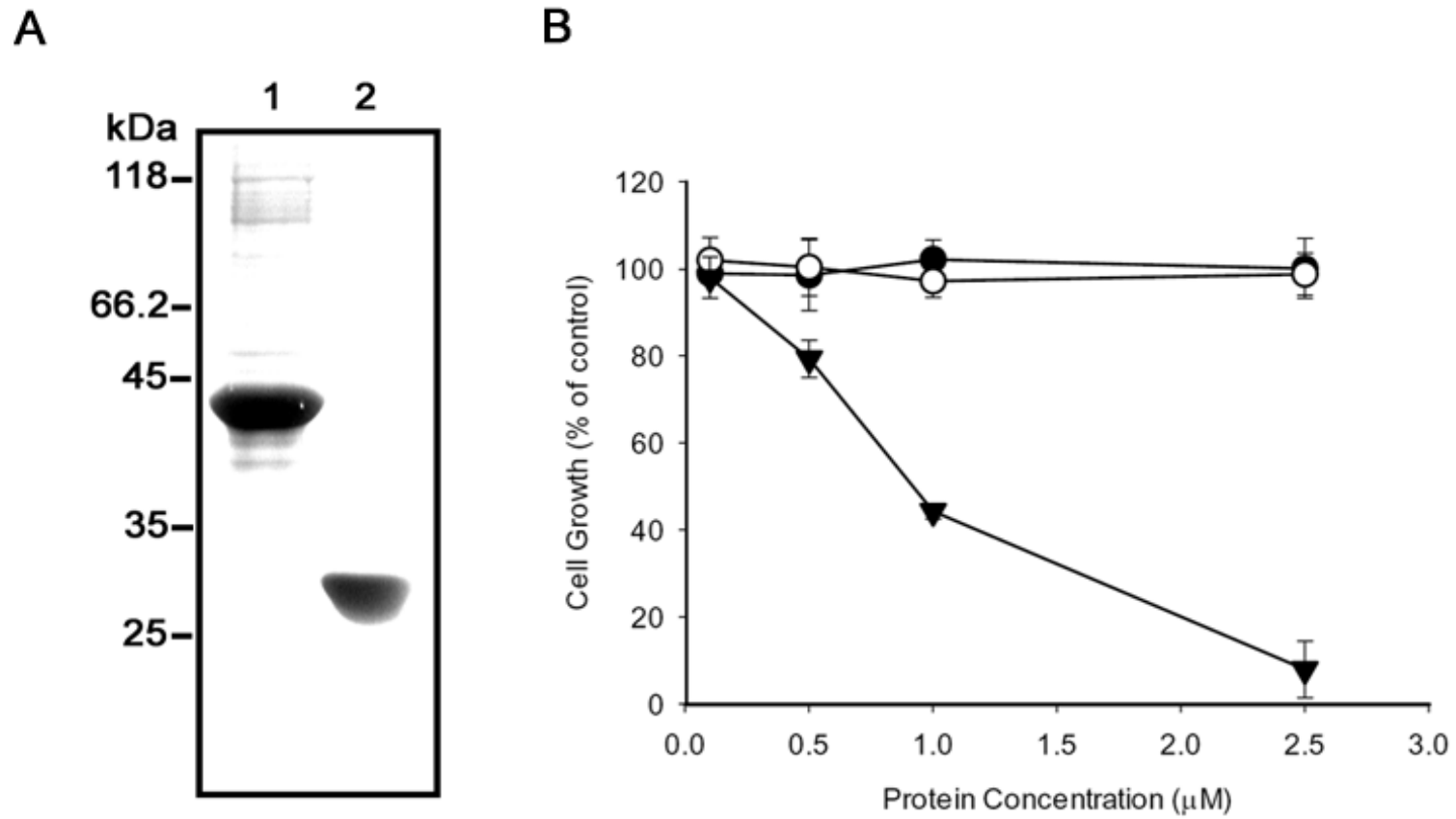

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Figure 2

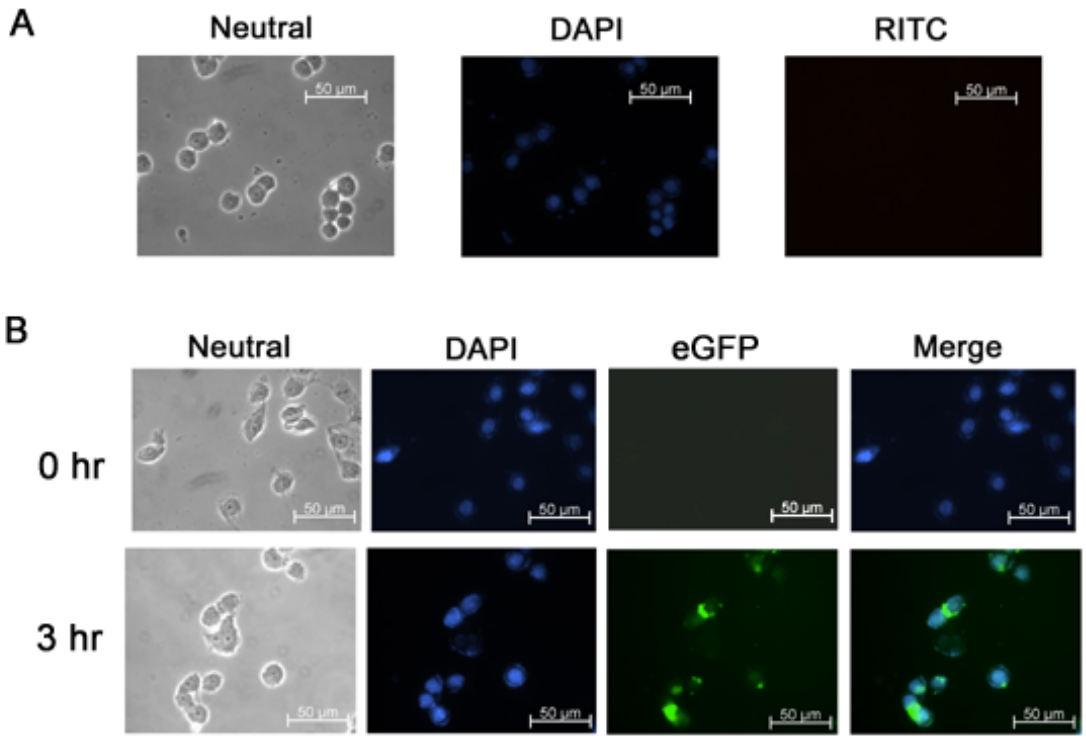

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Figure 2

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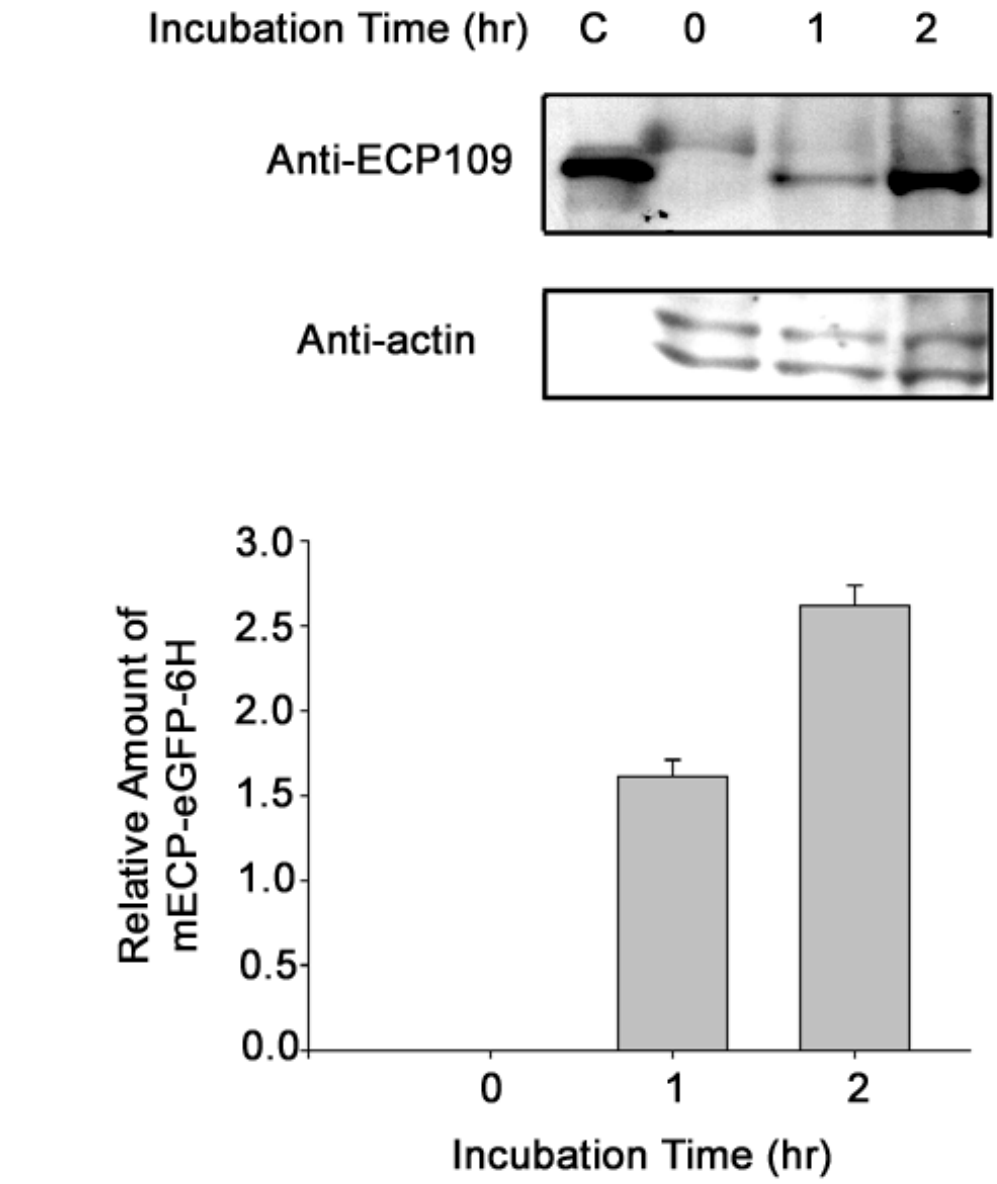

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Figure 3

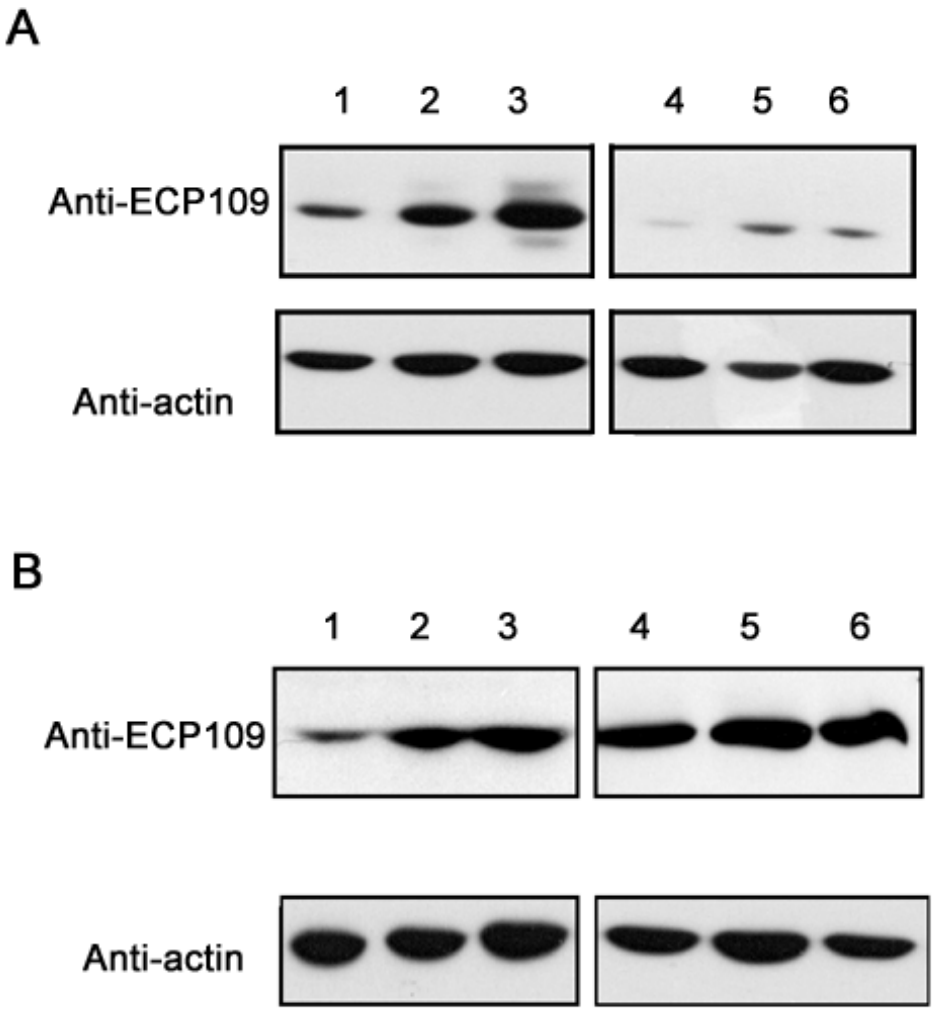

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Figure 4

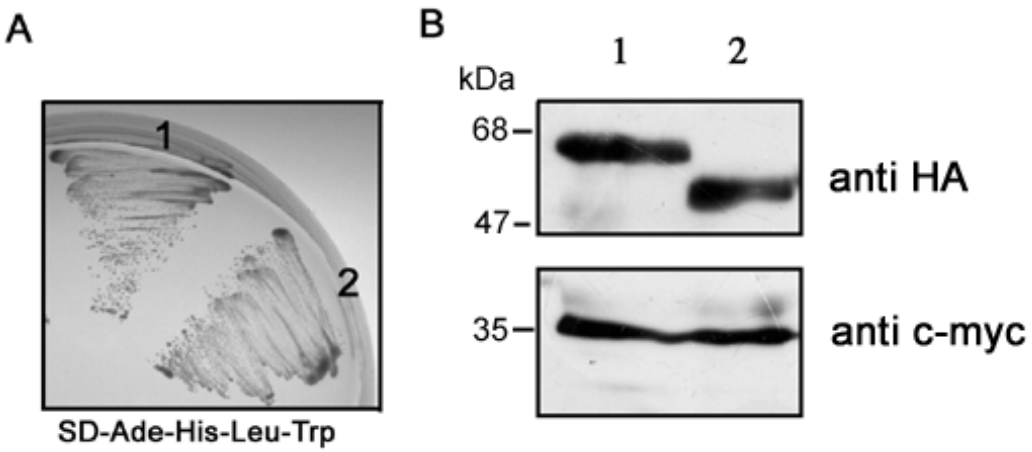

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Figure 5

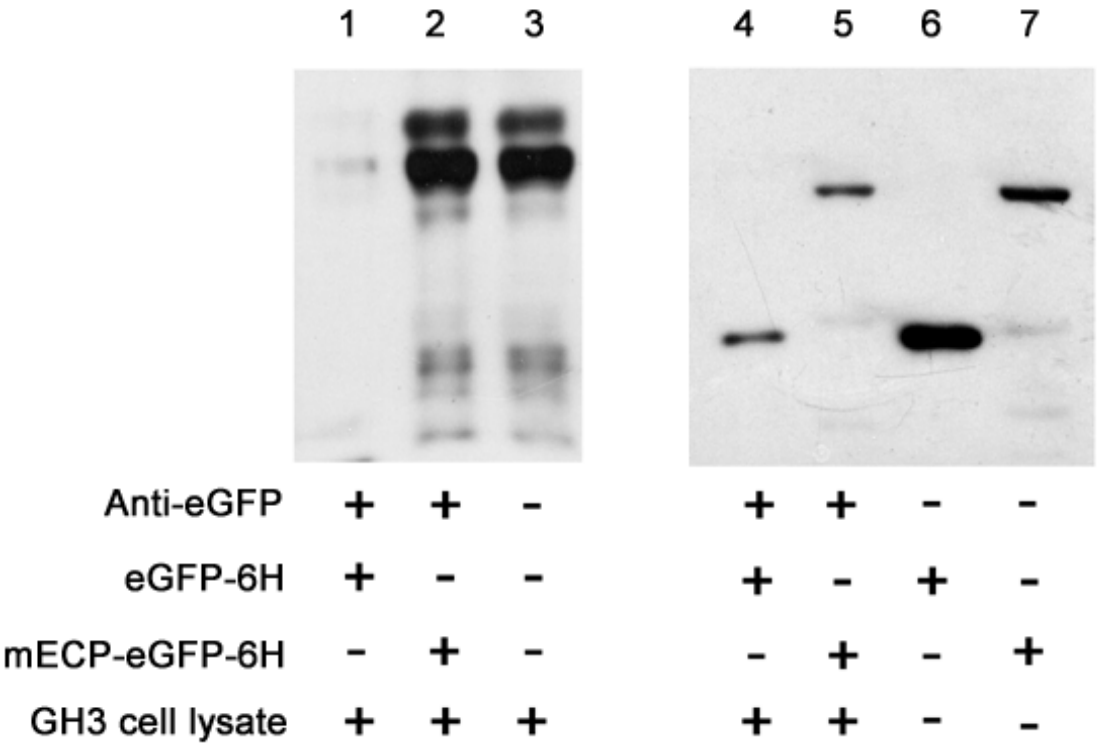

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Figure 6

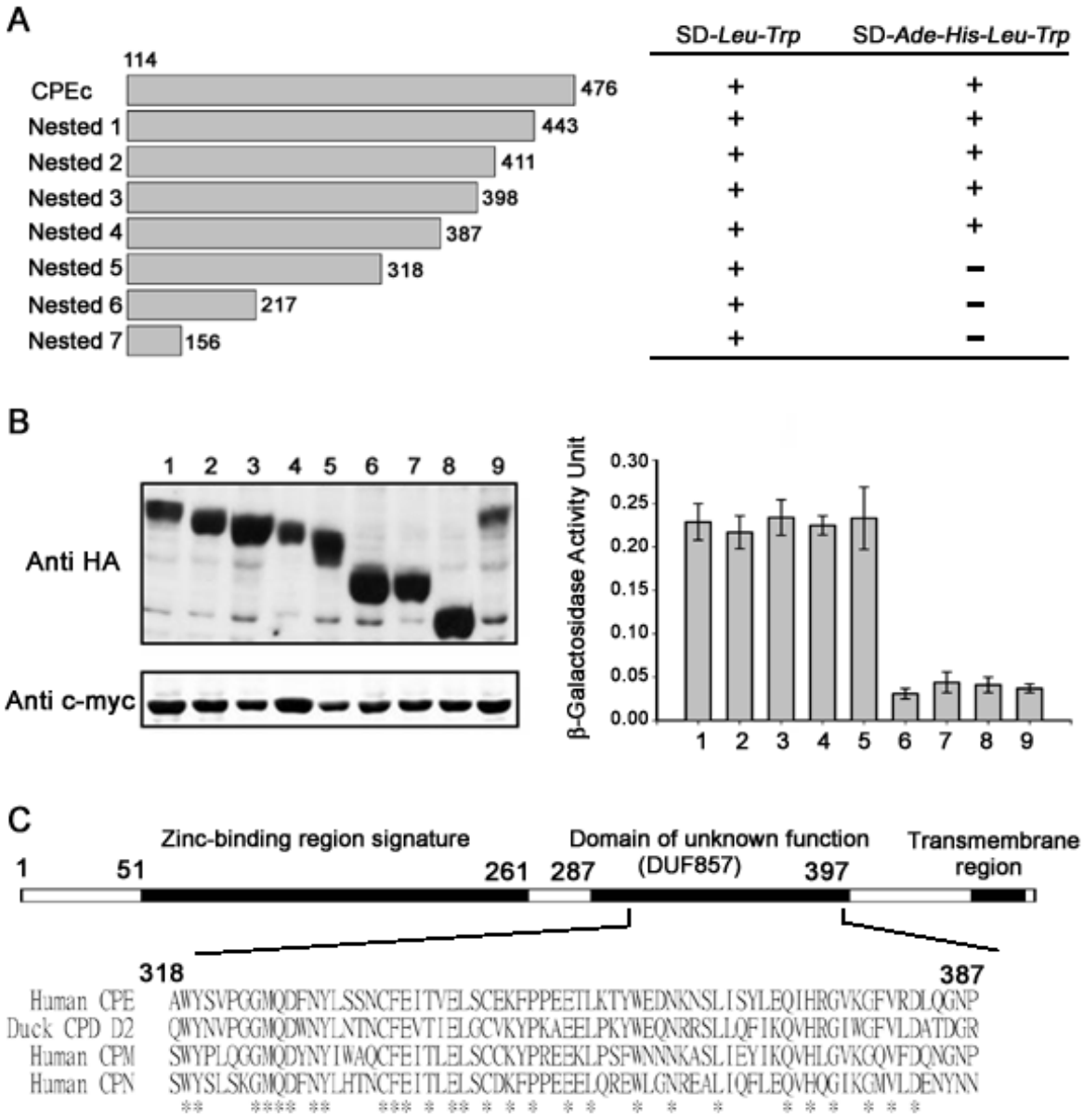

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Figure 7