High-level production and initial crystallization of a Fe65 PTB domain

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Fe65, a neuron-specific adaptor protein, has two phosphotyrosine binding (PTB) domains. The second PTB (PTB2) domain interacts with intracellular domain fragment (AICD) of amyloid beta precursor protein (APP). Recent studies suggested that the complex is composed of AICD and Fe65 transactivates genes that are responsible for neuronal cell death in Alzheimer's disease (AD). Therefore, a compound inhibiting the interaction between Fe65 and AICD can be a drug candidate to treat AD. However, it remains unclear how Fe65 recognizes AICD at a molecular level. Here, we report high-level production of the PTB2 domain of Fe65 in the baculovirus system. We found that the baculovirus system is an efficient method to obtain the Fe65 PTB2 domain, compared with the bacterial and mammalian expression systems. The purified recombinant protein was used for crystallization to determine its crystal structure helping to understand the molecular mechanism of Fe65-dependent signaling and to design its inhibitors.

Introduction

Alzheimer's disease (AD) is a neurodegenerative disease characterized by extracellular plaques and intracellular neurofibrillary tangles (NFTs), both of which are associated with amyloid beta precursor protein (APP)[9,11]. Consecutive cleavages of APP generate extracellular amyloid beta proteins (Aβ) and intracellular domain fragments (AICD) [12,15]. Secreted Aβ forms the extracellular plaque which initiates a cascade of events, leading to neuronal death and dementia[15].

Recently, it was reported that the presence of NFTs in neuronal cells is more closely linked to neurodegeneration than the amyloid plaque[5]. In AD, Tau undergoes hyperphosphorylation by glycogen synthase kinase-3β (GSK-3β) and becomes a main component of NFTs by forming twisted filaments, which alter its physiological functions in neurite morphogenesis[13]. Several reports suggest that the intracellular domain fragments of APP (AICDs) mediate transactivation of genes leading to hyperphosphorylation of Tau in the presence of the neuron-specific adapter protein Fe65[4]. Fe65, originally considered to be a transcriptional activator, contains two phosphotyrosine binding (PTB) domains[6]. The second PTB (PTB2) domain of Fe65

is responsible for its interaction with AICD in a tyrosine phosphorylation-independent manner[2,7]. A recent study suggested a mechanism, in which the complex composed of AICD and Fe65 transactivates genes after translocation of the complex into the nucleus[4]. Several target genes of the Fe65-dependent signaling pathway have been reported [1,8]. In particular, expression of GSK-3β is significantly upregulated by the AICD/Fe65 complex in the nucleus, resulting in hyperphosphorylation of Tau[8]. This previous study suggests that inhibition of the interaction between Fe65 and AICD will help to treat AD.

Although several three-dimensional structures of PTB domains have been determined, including the PTB domain of X11 which binds to AICD[16], they cannot be directly applied to determine the structure of the Fe65 PTB2 domain because of the low sequence homology among them. Therefore, a high resolution structure of the Fe65 PTB2 domain is required in order to better understand the molecular mechanism of the signal transduction involving Fe65 and AICD and to design inhibitors of this pathway. Although the PTB domains of Fe65 have been expressed in transfected mammalian cells to analyze their ability to bind APP[2], the expression level of these proteins is not high to apply the structural study.

As a first step toward determining the crystal structure of the PTB2 domain of human Fe65 protein, we report the overexpression, purification, and preliminary crystallization

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of this protein.

Materials and methods

Vector construction of pFASTBAC-HTc-GST

The vector pFASTBAC-HTc (Invitrogen) was modified to generate a vector expressing a GST-fusion protein. Prior to introduction of the *Glutathione-S-Transferase (GST)* gene, the hexa-His tag region in pFASTBAC-HTc was substituted with a deca-histidine tag plus the SmaI site using polymerase chain reaction (PCR), resulting in the vector pFASTBAC-10HTc. The primer sequences are as follows: 5'-GCGCCCGGGATGATGATGATGGTGATGGTGATGGT GATG-3' and 5'-CGCCCCGGGCATTACGATTACGATATC CCA-3'. Then, the GST gene, which was amplified from the GST-containing vector pGEX-4T1 by PCR, was introduced into the SmaI site of pFASTBAC-10HTc, generating the vector pFASTBAC-HTc-GST. The primers for amplification of *GST* gene were 5'-CGCCCCGGGATGTCCCC TATACTAGGTTA-3' and 5'-CGCCCCGGGGGATCCACGC GGAACCAGATCC-3'.

Fe65 PTB2 domain plasmid construction

The DNA fragment encoding the PTB2 domain region (536-662) was amplified from a human thymus cDNA library by PCR. The primers for this PCR were 5'-CCGGAA TTCAAAGAATGAGTTGGTCCAG-3' and 5'-CCGCCAAGC TTTCACAGACACTTCTGGTAGCGA-3'. We inserted the DNA fragment spanning the PTB2 domain region into the pFASTBAC-HTc-GST vector using EcoRI and HindIII sites, resulting in pFASTBAC-HTc-GST-PTB2. The identities of all DNA plasmids were confirmed by DNA sequencing analysis using the Thermo Sequenase II dye terminator cycle sequencing premix kit from Amersham Pharmacia Biotech.

Cell culture and transfection

The plasmid pFASTBAC-HTc-GST - PTB2 was transfected into SF-9 cells using CELLFECTIN reagent (Life Technology) according to the manufacturer's manual (Bac-to-Bac Baculovirus expression system, Life Technology). The recombinant virus was amplified in SF-9 cells in SF-900II serum-free medium (Life Technology). To produce the recombinant protein, SF-9 cells were maintained and cultured in 2 l of Grace medium supplemented with 20% SFM medium and 0.1% Pluronic F-68 reagent (Life Technology) as previously described[10]. Cells at a density of 3×10^6 cell

/ ml were infected at a multiplicity of infection of 1 - 5, and were harvested by centrifugation at 48 hr post-infection. The cells were stored at -70°C until use.

Cell disruption and purification of the PTB2 domain using Ni-NTA

The cells were resuspended in 50 ml of resuspension buffer[20 mM Tris buffer (pH 8.0), 200 mM NaCl, 2 mM 2-mercaptoethanol, and 0.5 mM phenylmethylsulfonyl fluoride (PMSF)]. Then, the resuspended cells were sonicated for 30 sec at 4°C and the cellular debris was removed by centrifugation at 12000 rpm for 30 min at 4°C. Subsequently, approximately 2 ml of Ni-NTA resin (Qiagen) was added to the supernatant (50 ml) in an empty column (Biorad, 2.5 cm x 12 cm) and the mixture was incubated for 1 hr at 4°C on a rolling mixer. The resin was recovered by flowing the slurry through the column, and washing with 100 ml of the resuspension buffer containing 20 mM imidazole. The bound fusion protein was eluted from the resin with resuspension buffer containing 200 mM imidazole. Fractions containing the fusion protein were analyzed on SDS-PAGE followed by staining to examine expression levels and purity.

TEV protease cleavage

Fusion protein-containing fractions eluted from the Ni-NTA column were pooled. One hundred μg of recombinant TEV protease was added per milligram of the fused protein, and the cleavage reaction was incubated in the presence of 5 mM 2-mercaptoethanol for 18 hr at 4°C.

Anion-exchange and size-exclusion chromatography

The cleavage mixture (10 ml) was diluted with distilled water (40 ml) and loaded onto Mono-Q HR (Pharmacia) (Fig. 3B). Unbound fractions were collected and concentrated into 2 ml with an ultrafiltration devise (5 kDa molecular cutoff, Millipore). The sample was then applied to a Hiload Superdex S-200 16/60 column (Pharmacia), equilibrated with 20 mM Tris (pH 8.0) buffer containing 200 mM NaCl and 2 mM 2-mercaptoethanol. The Fe65 PTB2 domain protein was isolated from the column and concentrated to 3 mg/ml with an ultrafiltration devise. The protein concentration was measured by the Bradford protein assay (Biorad) using bovine serum albumin as a standard[3].

Determination of N-terminal sequences

The purified protein was separated by SDS-PAGE under

reducing conditions, and electroblotted onto a poly-vinylidene difluoride membrane according to the instructions of the manufacturer (Millipore). The protein band was subsequently visualized by staining with amido black. The membrane was destained with 7% acetic acid, washed with distilled water and dried. The protein bands were cut into small pieces and subjected to sequencing by an Applied Biosystem 477A automated protein sequencer.

Crystallization screening

Initial crystallization trials were carried out by the sitting-drop vapor-diffusion method using Hampton Research Crystal Screen HT (Hampton Research). Each drop was formed by mixing equal volumes (0.5 μl) of protein solution and reservoir solution which then equilibrated via vapor diffusion over 50 μl of reservoir solution at 14°C.

Results and Discussion

Vector features

It was reported that X11 PTB domain and Dok1 PTB domain were effectively overexpressed in the bacterial expression system[14,16]. Initially, we attempted to express the PTB2 domain of Fe65 using diverse expression vectors in the bacterial expression system. However, the protein was not successfully expressed in a soluble form (data not shown). The baculovirus/insect cell system was selected to overexpress the Fe65 PTB domain because of the many ad-

vantages of this system for expressing recombinant proteins that originate from eukaryotic cells.

We chose the pFASTBAC-HTc vector (Invitrogen) since this vector was designed for the Bac-to-Bac expression system (Invitrogen) which is a fast method to construct recombinant baculovirus. The resulting pFASTBAC-HTc-GST vector contains a deca-His affinity tag, GST, the TEV protease recognition site and an extensive multiple cloning site (MCS); Fig. 1 depicts this vector design along with a schematic diagram of the expressed proteins and the sequence between the deca-His tag and the MCS. Both the deca-His tag and GST protein can be used for affinity purifications using Ni-NTA and Glutathione affinity chromatographies, respectively.

Expression of recombinant Fe65 PTB2 domain

The DNA fragment encoding amino acids 536-662 of Fe65 was ligated into the pFASTBAC-HTc-GST vector, generating pFASTBAC-HTc-PTB2. The recombinant baculovirus was produced and amplified according to the Bac-to-Bac method (Invitrogen). SF-9 cells were cultured and infected by the amplified baculovirus. To examine the initial expression level, the recombinant proteins were initially purified from the cell fraction using both Ni-NTA and Glutathione affinity chromatographies. We found that Ni-NTA affinity chromatography was more effective at the initial purification step than Glutathione affinity chromatography because endogenous GST significantly bound to the
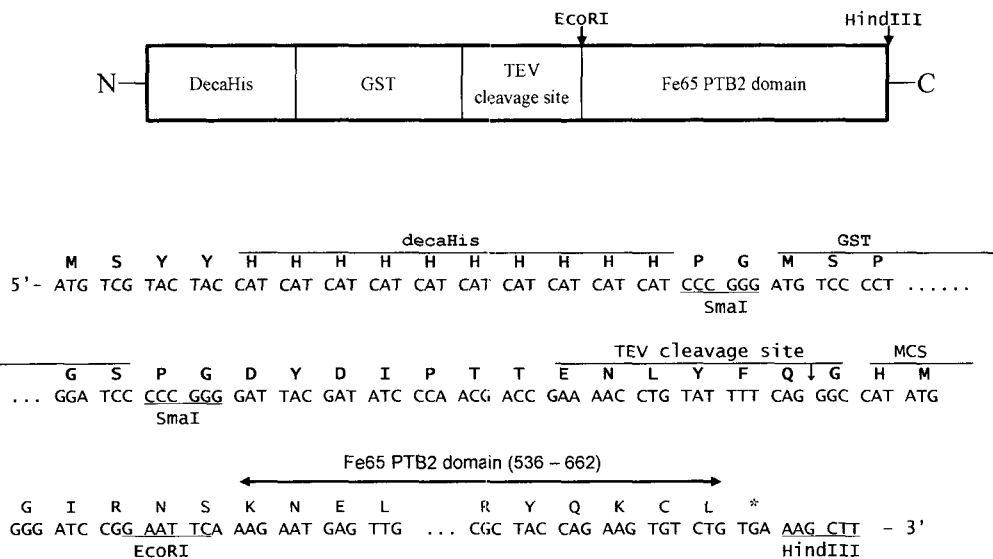
Fig. 1. Map of the pFASTBAC-HTc-GST-PTB2 vector. *Top*, a bar diagram of the protein expressed from the pFASTBAC-HTc-GST-PTB2. *Bottom*, the DNA and protein sequences are shown with the restriction enzyme sites mentioned in "Materials and Method". The TEV protease cleavage site is indicated by an arrow.
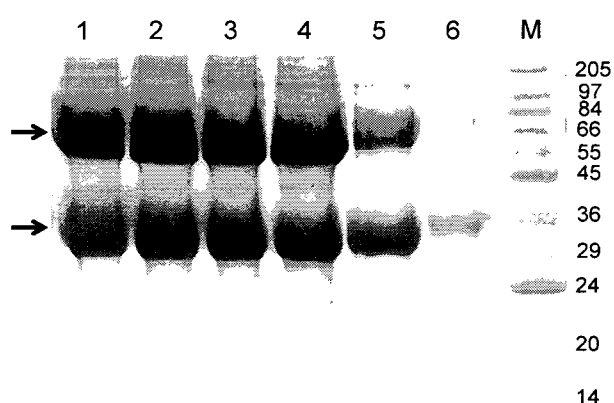
Fig. 2. SDS-PAGE gel showing the eluted fractions from the Ni-NTA agarose column. The arrows indicate the GST-fused Fe65 PTB domain bands. Fifteen μl were taken from each fraction (1 ml) and loaded onto an SDS-PAGE gel for analysis. The gel was stained with Coomassie Blue. Protein molecular weight standards are shown in lane M.

resin during Glutathione affinity chromatography. A high purity of the protein was achieved by one-step metal affinity chromatography, as visualized by Coomassie-stained SDS-PAGE gels (Fig. 2). Unexpectedly, two bands with different sizes (36 kDa and 75 kDa) appeared on the SDS-PAGE gel, but both bands turned out to be GST-fused PTB2 domain during subsequent purification (See below). The estimated yield of the GST-fused PTB2 domain was 2 mg per 1 liter of cell culture.

Purification of recombinant Fe65 PTB2 domain

To release the Fe65 PTB2 domain from its fusion partner, a TEV protease was used. Its recognition site was located between the GST and PTB2 domains. Overnight incubation with recombinant TEV protease resulted in complete digestion at the TEV protease recognition site, yielding two fragments (24 kDa band for GST and 10 kDa for Fe65 PTB2 domain) as judged by SDS-PAGE (Fig. 3A). The reaction mixture was diluted 4-fold with distilled water to reduce the salt concentration, and then the mixture was loaded into a strong anion exchange chromatographic column. The unbound fractions were collected since the PTB2 domain protein does not bind to the column (Fig. 3B). Most of the GST protein was tightly bound to the anion exchange column (Fig. 3B). The unbound fractions were concentrated and then loaded into a size-exclusion chromatographic column. The observed molecular weight of the PTB2 domain protein on the size exclusion column was matched with the calculated molecular weight of the
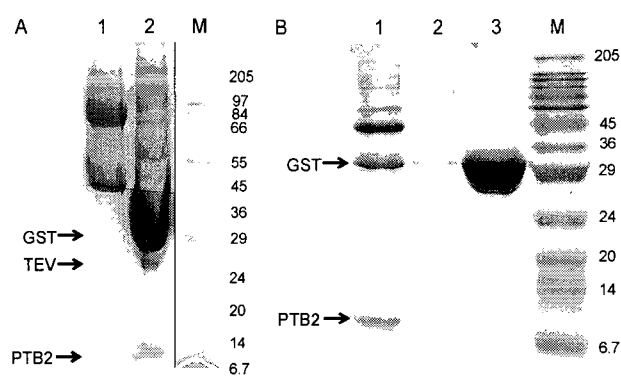
Fig. 3. TEV protease cleavage and anion exchange chromatography. (A) The eluted factions from the Ni-NTA column were pooled and analyzed on an SDS-PAGE gel, as shown in lane 1. GST, the PTB2 domain, and TEV protease, which were generated by TEV protease digestion of the sample shown in lane 1, are shown in lane 2. Protein molecular weight standards are shown in lane M. (B) The sample shown in lane 2 of Fig. 3A was loaded into a Mono-Q anion exchange column. The unbound fraction (lane 1), washed fraction (lane 2) and bound fraction (lane 3) are shown, together with protein molecular weight standards (lane M).

monomeric species, indicating that the recombinant PTB2 domain protein was correctly folded and that the protein was not a large molecular aggregate (Fig. 4).

Fractions containing pure PTB2 domain were pooled and concentrated to 3 mg/ml. The purity of the final purified PTB2 domain protein was ~95% as determined by SDS-PAGE and coomassie staining. The identity of the resulting protein was confirmed by amino-terminal amino acid sequencing. The final yield was about 0.3 mg of purified Fe65 PTB2 domain per 1 liter of cell culture.

Initial crystallization

The purified protein was used to screen for crystallization conditions with a high-throughput crystallization screening kit at various temperatures. At present, sphere-shaped initial crystals were obtained in a reservoir solution containing 0.2 M ammonium sulfate, 0.1 M MES (pH 6.5), and 30% polyethylene glycol monomethyl ether 5000 (Fig. 5). This result demonstrates that the recombinant protein is correctly folded, because only properly folded proteins can be crystallized. However, the crystals have not been able to be characterized because of their low quality. The crystallization conditions needs to be optimized before the crystals can be characterized.

In conclusion, we have presented an efficient procedure for the production of a large quantity of the Fe65 PTB2
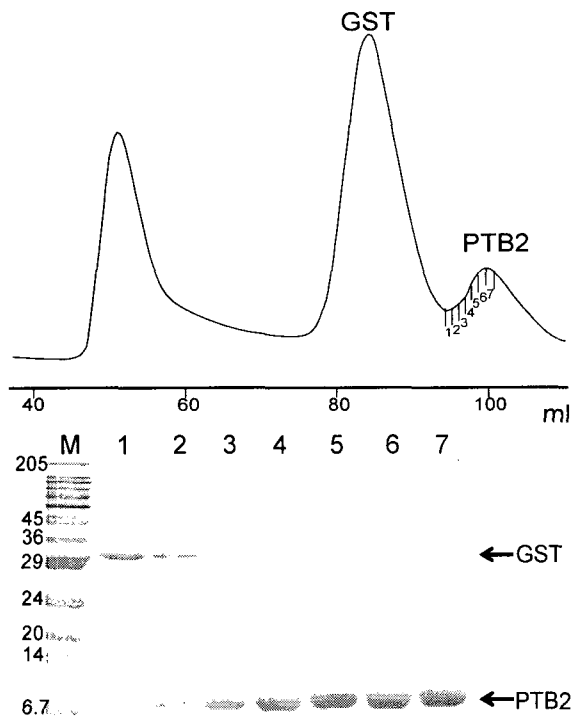
Fig. 4. Size exclusion chromatography. The concentrated un-
bound fractions from Mono-Q anion exchange column
were loaded into Hiload Superdex S-200 HR 16/60 in
20 mM Tris (pH 8.0) buffer containing 200 mM NaCl
and 2 mM 2-mercaptoethanol. The major protein is in-
dicated on each peak. Twenty μl from each fraction
(1.5 ml) were analyzed by SDS-PAGE (bottom). Each
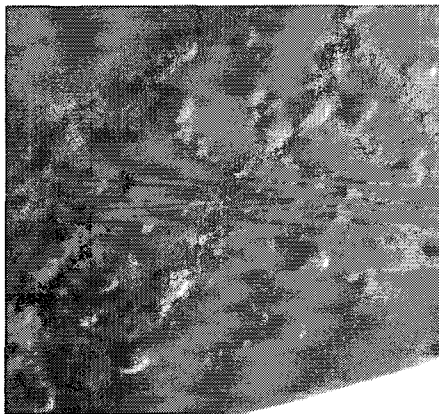lane number (1-7) corresponds to the fractions in-
dicated on the chromatogram.

Fig. 5. Initial crystals of the Fe65 PTB2 domain. The size of a
single crystal is approximately 0.02 x 0.02 x 0.01 mm.

domain in the baculovirus/insect cell system. Initial crystals
were obtained from the purified Fe65 PTB2 domain, and
the protein sample is now being used to optimize crystal-
lization conditions, the first step toward determination of
the three-dimensional structure of this protein.

Acknowledgement

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초록 : Fe65단백질의 한 PTB 도메인에 대한 과발현 및 초기 결정화

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신경세포에 특이적으로 발현되는 단백질인 Fe65는 두 개의 phosphotyrosine binding(PTB) 도메인을 가지고 있다. 두번째 PTB(PTB2) 도메인은 아밀로이드 베타 전구 단백질(APP)의 세포질 도메인 조각(AICD)와 결합한다. 최근 연구 결과들은 AICD와 Fe65로 이루어진 결합체가 알츠하이머병에서 신경세포를 죽게 하는 유전자를 발현한다고 제시하고 있다. 따라서 Fe65와 AICD의 결합을 방해하는 화합물은 알츠하이머병을 치료하는데 후보물질이 될 수 있다. 하지만 AICD와 Fe65와 관련된 신호전달에 대한 분자적 기전은 잘 알려져 있지 않다. 이번 연구에서는 Fe65의 PTB2 도메인을 baculovirus 시스템에서 과발현시킨 결과를 보고한다. 세균 및 척추동물 세포를 이용한 시스템과 비교했을 때, baculovirus 시스템이 훨씬 효과적이다는 것을 발견했다. 정제된 재조합 단백질을 이용하여 초기 결정을 얻었다. 결정을 이용하여 앞으로 밝힐 3차원 구조는 Fe65관련 신호전달체계에 대한 분자 기전 및 이에 대한 저해제 개발에 큰 도움을 줄 것이다.