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PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Pakistan Journal of Biological Sciences 3 (10): 1740-1742, 2000

Identification of Human Serum Proteins Which Interact With Alzheimer's Amyloid βA4 Protein

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Abstract: Alzheimer's amyloid βA4 protein fused with glutathione S-transferase (GST) was highly expressed using a strong prokaryotic expression system in *Escherichia coli*. The expressed protein had expected molecular mass on SDS-PAGE and appeared exclusively immunoreactive with antibody specific for βA4 epitope. This recombinant protein was purified with a combination of urea solubilization and ion exchange chromatography. To identify the human serum proteins which interact with βA4, affinity columns were prepared by immobilizing GST-βA4 and GST respectively. Using the affinity columns and human serum, we have observed an interaction of βA4 with serum proteins. Two proteins of Mr 45 and 15 kDa were identified on SDS-PAGE to be involved in the interaction. Our demonstration of the ability of βA4 to interact with serum protein strongly support the notion that such an interaction may underlie with the biological function of βA4 *in vivo*.

Key words: Alzheimer's amyloid βA4 protein, interaction, human serum proteins

Introduction

Amyloid βA4 protein is a normal 4 kDa derivative of a large transmembrane glycoprotein, amyloid precursor protein (APP). It is found in an aggregated, poorly soluble form in extracellular amyloid depositions in the brains and leptomeninges of patients with Alzheimer's disease (AD) (Selkoe, 1991).. In contrast, βA4 is also found in a soluble form in human cerebrospinal fluid (CSF), in serum of both healthy and AD subjects (Shoji *et al.*, 1992; Scheubert *et al.*, 1992). Little is known about the metabolic fate of soluble βA4 and the mechanisms of βA4 aggregation and plaque formation *in vivo*. In particular it is unclear whether βA4, once secreted, is free in biological fluids or associated with other proteins and thus transported and metabolized with them. Such knowledge is a prerequisite for understanding the biological action of βA4 and in turn the pathogenesis of AD.

In this study we have designed an experimental approach to detect the interaction of βA4 with human serum proteins that may provide the basis for biological action of βA4.

Materials and Methods

Material: Restriction endonuclease and modification enzymes, reagents for PCR and ligation kits were purchased from Takara Shuzo, Japan. Glutathione sepharose 4B resin and reduced glutathione were from Sigma. Monoclonal antibody specific for βA4 1-10 of βA4 was prepared as described (Shinoda *et al.*, 1990). Unless indicated otherwise, all reagents used in this study were of analytical grade and obtained from Wako Pure Chemical Industries, Japan.

Plasmid construction and bacterial expression of GST - βA4: The cDNA fragment encoding residues 591-639 of APP695 that included the C-terminal 43 amino acids of βA4 was amplified by pCR from APP695 using the reverse primer, 5' GGA GGA GAT CTC TGA AGT GAA GAT GGA TGC AGA ATTC - 3', PCR product was digested with Hind III, the termini made blunt-ended with klenow, and again digested with Bgl II for in frame insertion into a recombinant expression vector P^{GEX-3X} (Pharmacia) at the downstream of the lac promoter (Fig. 1). The resulting plasmid, $P^{GE-βA4}$ was transferred into DH5 strain of *E. coli* and the fusion protein, GST-βA4 was expressed efficiently. For optimum expression, *E. coli* DH5 cells carrying pGE-βA4 were grown at 37°C and then induced with isopropyl β-D-thiogalacopyranoside (IPTG) for 3 h.

Purification of Recombinant GST - βA4: Bacterial cells were harvested by centrifugation and were suspended in STET buffer (50 mM Tris, pH 8.0; 25 mM EDTA, 8% sucrose and 0.7% Triton x 100) and sonicated. Following centrifugation, the pellet was solubilized in NTE buffer (100 mM NaCl, 10 mM Tris, pH 7.4 and 1 mM EDTA) containing 2 M urea. The soluble fraction was loaded onto ion exchange column (Q-sepharoses Pharmacia) equilibrated with TE buffer containing urea and eluted with NaCl step-wise. GST-βA4 fraction eluted with .4 M NaCl, and was dialyzed against 50 mM Tris-HCl, pH 7.5. The purified protein was characterized by SDS-PAGE followed by Coomassie blue staining.

Analysis of the interaction of βA4 protein with proteins in human serum: To study the interaction, affinity chromatography was employed and g'utathione sepharose 4B was used as resin. Resin bound to GST-βA4 and resin bound to GST were prepared as described (Smith *et al.*, 1988) and were packed separately on to 2 ml minicolumns (Pierce). Aliquots of 1 ml of freshly prepared human serum

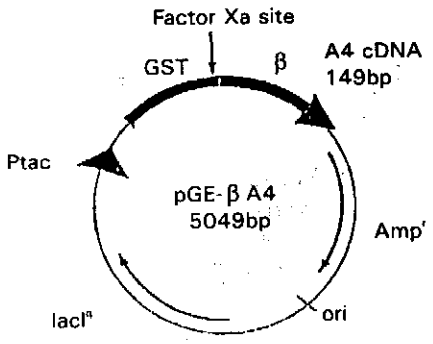

Fig. 1: Schematic representation of the PGE-βA4 construct. cDNA fragment for βA4 encoding residues 591-639 of APP695 is fused with a GST fusion partner under control of tac promoter. Also indicated are the ampicillin resistance gene (Amp ^r), the origin of replication (ori).

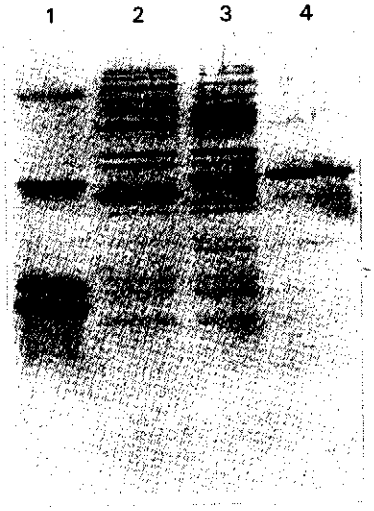

Fig. 2: Expression and purification of GST-βA4. Protein extract were compared on a 15% Trisglycine SDS-polyacrylamid gel stained with Commassie brilliant blue. Land 1, molecular mass marker consisting of bovine serum albumin (67 kDa), carbonic anhydrase (29 kDa), myoglobin (17 kDa) and cytochrome C (12 kDa), lane 2, 25 μg uninduced bacterial extract, lane 3, 25 μg bacterial extract induced with 0.1 mM isopropyl thiogalactoside for 3 h.,lane 4, purified GST-βA4 recombinant protein after elution from sepharose column.

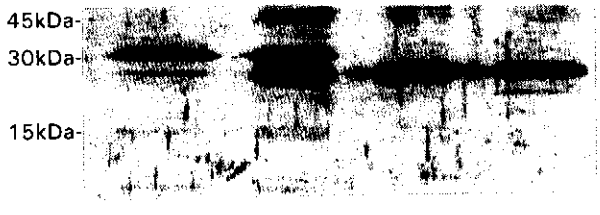

Fig. 3: Silver stained gel of reduced glutathione sepharose 4B column containing bound GST-βA4 or GST incubated with human serum. Lane 1, GST-βA4, and lane 4, GST, lane 2, fraction from a column containing bound GAST-βA4 3, fraction from a column containing bound GST. Positions of molecular mass markers (kDa) are shown in the left.

diluted 2-fold in column buffer (20 mM Tris-HCl, pH 7.5) were added to the columns and incubated batch-wise with gentle agitation overnight. The columns were washed with 20 vol. of column buffer and eluted with 3 ml of 10 mM reduced glutathione in column buffer. The eluted fractions were then concentrated using a 3 kDa cut-off YM3 Diaflo membrane (Amicon), resuspended in the sample buffer and subjected to SDS-PAGE using 18% acrylamide.

SDS-PAGE, immunoblot and silver staining analysis: SDS-PAGE, immunoblot and silver staining analysis were performed as described previously (Lamelli, 1970; Merril *et al.*, 1981). For immunoblot, the membranes were incubated with anti-βA4 antibody, then incubated with secondary antibodies conjugated with peroxidase.

Results and Discussion

We engineered a construct that allowed us to produce in *E.coli* the amyloid βA4 protein, encoding residues 591-639 of APP695, fused to GST using the P^{GEX-3X} expression plasmid. On induction with 0.1 mM IPTG, plasmid - βA4 containing cells were found to have an additional 30 kDa coomassie blue stainable band when SDS-PAGE was carried out on induced-cell lysates (Fig. 2). This band could not be seen on uninduced or control lysates. A 3 h induction was found to be optimal for expression and was not improved by longer incubation periods; indeed electron microscopy of cell pellets obtained after longer incubation periods showed significant lysis and cell death compared with controls (results not shown). In this system, GST-βA4 was expressed to levels of 30-50% of the total bacterial protein. Since the fusion protein was over expressed in the form of insoluble inclusion bodies, bacteria was solubilized in urea after washing with STET buffer. We took advantage of this insolubility to eliminate most of the soluble protein in the first step of its purification. Then the expressed protein was purified by ion exchange chromatography (IEC). The purified protein was found to be homogeneous, as assessed by SDS-PAGE, followed by Coomassie blue staining (Fig. 3). To identify this fragment as βA4 fused with GST, we used the anti-βA4 antibody. By Western blot analysis, this antibody cross reacted with the purified protein. On the other hand, GST was prepared as described (Smith *et al.*, 1988).

In order to investigate whether serum proteins were able to interact with βA4, we employed an experimental approach based on the reversible binding of the GST - βA4 and resin-bound GST were prepared as mentioned earlier (Smith *et al.*, 1988). Aliquots of human serum were incubated in mini-columns containing resin-bound GST-βA4. After the addition of reduced glutathione, the presence of proteins in the eluates was tested by silver staining (Merril *et al.*, 1981), the most sensitive technique for the detection of protein. As shown in Fig. 4; whereas no signal was detected in the eluate from column containing GST-bound resin, one intense bound with Mr 45 kDa and one less intense bank with Mr 15 kDa were found in the eluate from the column containing GST-βA4 bound resin. This result indicated that βA4 moiety was responsible for the interaction observed.

A variety of histological, molecular genetic, and in vitro and *in vivo* studies have provided evidence to believe that βA4 is central to the pathogenesis of AD (Selkoe, 1993). Under neuropathological conditions such as Alzheimer's diseases this peptide has a greater propensity to self aggregate (Selkoe, 1991) and contribute to the neurotoxicity (Pike *et al.*, 1991). The neurotoxicity of βA4 is also dependent on its aggregation state and requires the assembly of βA4 into

amyloid fibrils (Lorezo *et al.*, 1994). Assembly of βA4 into fibrils may be catalyzed by several factors that bind to βA4 and increase its aggregation *in vitro* including the heavy metals, Zinc and Aluminum (Bush *et al.*, 1994; Fasman *et al.*, 1995). Thus studies of βA4 have traditionally emphasized its toxicity. Since βA4 is also normally released from neuronal cells in a soluble from (Shoji *et al.*, 1992; Scheubert *et al.*, 1992) researchers have searched for physiological roles of the peptide. The physiological functions of βA4 are not well understood, although several reports indicated an array of biological activities, including the trophic response (Yuanker *et al.*, 1990), neuronal cell signalling (Zhang *et al.*, 1994; Luo *et al.*, 1996), compromising the cholinergic neuronal function (Pedersen *et al.*, 1996) etc. However, the physiological mechanism of the biological action of βA4 and the mechanism of βA4 aggregation and plaque formation is still unknown. Specific interactions between proteins form the basis of many essential biological actions. In our experiment, we have observed an interaction of βA4 with the human serum proteins in human serum. The observation supports the idea that such an interaction may underlie with the mechanism for the action of βA4.

In conclusion, we have successfully expressed and characterized βA4 protein as fusion with GST in bacteria. This recombinant GST-βA4 would be a valuable resource to study the binding protein in CSF, serum or brain. We have clearly shown that βA4 is able to interact with the proteins in human serum. Two proteins with Mr 45 and 15 kDa were identified to be involved in the interaction. It is expected that βA4 binding proteins found in this experiment may provide important knowledge to explain the function of βA4 and to add tesserae to the puzzling scenario of AD.

Acknowledgements

This work was supported in part by Grants-in-Aid from the Ministry of Education, Science and Culture of Japan.

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