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Cross-linking of β-amyloid protein precursor catalyzed by tissue transglutaminase

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Abstract

Alzheimer's disease is characterized by progressive dementia, cortical atrophy with synaptic loss, and the accumulation of neurofibrillary tangles and senile plaques containing β-amyloid. The β-amyloid protein precursor (β-APP), may normally be involved in cell adhesion related to synaptic maintenance. Loss of synapses correlates with dementia, suggesting that synaptic deficits may underlie the disease. Synapse stability may depend on the action of tissue transglutaminase (tTG), an enzyme capable of crosslinking large, multi-domain extracellular glycoproteins, that is active and present at synapses. We now show that β-APP is a substrate for tTG in vitro that results in dimers and multimers by silver staining and immunoblotting. This novel post-translational modification suggests further roles for β-APP in synaptic function as well as in Alzheimer's disease.

Key words: Transglutaminase; β-Amyloid protein precursor; Synapse; Alzheimer's disease; Protease; Serpin

1. Introduction

β-APP, a 106–110 kDa glycoprotein with three major isoforms, APP_{695}, APP_{751}, and APP_{770}, is encoded by a single gene [1–3]. The larger 751 and 770 isoforms contain the Kunitz protease inhibitor domain (KPI), lacking in the APP_{695} isoform [3–5]. β-APP undergoes extensive post-translational processing including glycosylation, sulfation, and proteolytic cleavage. These molecular changes have functional significance related to cell adhesion, regulation of neuronal growth, and protease inhibition [6]. Although controversial, APP_{770} and APP_{751} are the predominant forms of the protein in the brain [7], whereas in the peripheral nervous system, all three are found equally.

Tissue transglutaminase (EC 2.3.2.13; tTG) catalyzes the calcium-dependent intermolecular cross-linking of proteins via glutamine and lysine residues, both present in β-APP. Cross-linking involves an acyl-transfer reaction where the γ-carboxamide groups of peptide-bound glutamine residues act as acyl donors to the acyl acceptors, primary amino groups of lysine or other compounds, forming either ε-(γ-glutamyl)lysine or (γ-glutamyl)polyamine bonds. These covalent bonds are resistant to proteolysis by chemical means, including SDS, urea, guanidine, and reducing compounds [8]. tTG is ubiquitous and serves many biological functions including the formation of fibrin in blood clotting [9], rigid cross-linking of proteins as in erythrocyte membranes [10], in senile cataract formation in the optic lens [11], and in the cornified envelope of human epidermal keratinocytes [12].

Evidence strongly suggests that β-APP functions as a cell adhesion molecule in the nervous system [13–18]. A number of extracellular matrix (ECM) molecules such as collagen, laminin, fibronectin, and thrombospondin act as substrates for tTG [19,20]. After cleavage by the secretase enzyme, β-APP becomes an extracellular glycoprotein and, therefore, might also be a natural substrate for tTG. In this study, we show that guinea pig liver tTG catalyzes the in vitro crosslinking of purified recombinant β-APP_{770} into homodimers and multimers.

2. Materials and methods

Purified recombinant β-APP_{770} from *E. coli*, was a generous gift from Dr. B.D. Greenberg (Cephalon, Inc., West Chester, PA). Guinea pig liver tTG (Sigma, St. Louis, MO) was resuspended in 50 mM Tris-HCl buffer, pH 7.2, 0.9% NaCl at 1 μg/μl. All procedures were carried out on ice or at 4°C. The reaction mixture consisted of 3 μg tTG, 60 ng β-APP_{770}, 6 mM $CaCl_2$, protease inhibitor 'cocktail' (100 μg/ml soybean trypsin inhibitor, 5 mg/l leupeptin, 5 mg/l pepstatin, 135 KIU/ml aprotinin), in 50 mM Tris-HCl, pH 7.2, 0.9% NaCl, in a final reaction volume of 20 μl. The reaction was allowed to proceed at 37°C for 1 h, and then stopped by the addition of 30 mM EDTA in Tris buffer, pH 7.2. The reaction mixture was diluted with SDS-containing sample buffer, boiled at 100°C for 5 min, and then subjected to SDS-PAGE on pre-cast 4–12% Tris-Glycine gradient gels (Novex, San Diego, CA). Afterwards, the immobilized proteins were subjected to electrotransfer onto nitrocellulose membranes overnight at 4°C, and blocked for one hour in 5% bovine serum albumin (Sigma) in PBS/Tween-20 to eliminate nonspecific binding. Enhanced chemiluminescence Western blotting (ECL,

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Abbreviations: AD, Alzheimer's disease; β-APP, beta-amyloid precusor protein; KPI, Kunitz protease inhibitor domain; tTG, tissue transglutaminase; SDS, sodium dodecyl sulfate; ECM, extracellular matrix.

A preliminary account of these results has been presented in abstract form at the Marion Merrell Dow/Scientific Education Partnership Symposium, Molecular Mechanisms of Degenerative Diseases, Kansas City, MO, May, 1994.

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Amersham, Arlington Heights, IL; DuPont, Boston, MA) was performed using monoclonal anti-β-APP primary antibody (clone 22C11; Boehringer Mannheim, Indianapolis, IN) and goat, anti-mouse secondary antibody linked to horseradish peroxidase (Amersham). Other membranes were immunoblotted with anti-GID polyclonal antibody specific for residues 175–186 of β-APP (generous gift of Dr. Saitoh, UCSD, La Jolla, CA). Membranes were developed as instructed by manufacturers and then exposed to radiographic film (XAR-OMAT, Kodak, Rochester, NY).

For the next experiments, eight reaction mixtures, representing the eight time points, in duplicate, were prepared as described in Fig. 1, and incubated in a 37°C dry bath for appropriate amounts of time. They were subsequently placed at 4°C, and the reaction halted by the addition of 5 mM EDTA in Tris, pH 7.2. After the final tube completed incubation, SDS-containing sample buffer was added to each mixture. The mixtures were boiled at 100°C for 5 min, and separated by SDS-PAGE on a 4–12% pre-cast Tris-Glycine gel. Gels were stained with silver [21] and analysed by laser densitometry (Pharmacia Ultroscan). Each gel was scanned three times, and error bars were derived from this data. Band densities were plotted as a function of time using SigmaPlot (Jandel, San Rafael, CA).

3. Results

In our effort to further characterize the function and interactions of β-APP which might be relevant to Alzheimer's disease pathogenesis, we found that tTG is able to catalyze cross-linking of β-APP$_{770}$ in vitro. In the presence of β-APP, tTG and calcium, we found both a dimeric form, approximately 212 kDa, and higher kDa β-APP multimers that remained at or close to the top of the gel after SDS-PAGE (Fig. 1, lane 4), in addition to the β-APP monomer (106 kDa). The 212 kDa band, and the β-APP multimers (≈424 kDa), were absent when either β-APP or tTG were incubated with calcium alone, but not combined (Fig. 1, lanes 1–3). Similar results were obtained for purified recombinant human β-APP$_{695}$ (not shown). The monomeric β-APP$_{770}$, dimeric β-APP, and the multimeric β-APP bands were recognized by monoclonal antibody against the N-terminal region of β-APP (clone 22C11). A polyclonal anti-GID antibody, specific for residues 175–186 of β-APP [14], also recognized precisely the same pattern of bands as the 22C11 antibody (Fig. 1, lanes 5–7). We included protease inhibitors in the reaction so that the disappearance of the monomeric β-APP band at 106 kDa could not be attributed to protease action.

Having obtained these results, we determined if a substrate:product relationship of monomers to insoluble β-APP dimers and multimers existed by incubating β-APP$_{770}$, tTG and calcium for various times from 0–120 min (Fig. 2a, lanes 2–10). A monomeric 106 kDa band was clearly observed (lane 2) which decreases in intensity with time (lanes 3 through 10) as higher M_r forms increase. No bands at 212 kDa, or at the level of multimers, were noted at zero time, while progressive increase in the intensity of these protein bands with time was observed (lanes 2 through 10). In order to better quantify the reaction between 0 and 10 min, similar experiments were carried out for time points from 2 to

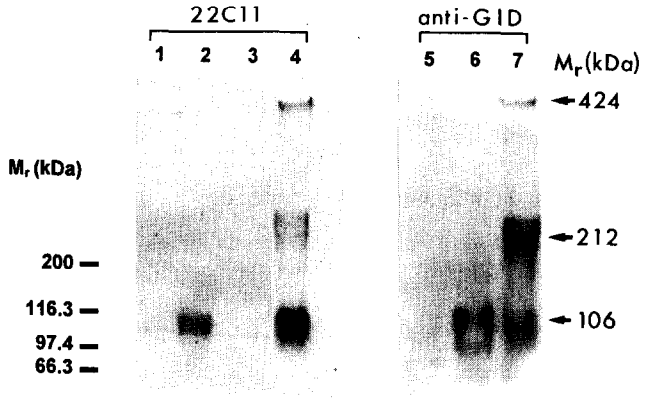

Fig. 1. Tissue transglutaminase cross-linking of β-APP$_{770}$ detected by immunoblotting. Lanes 1–4 immunoblotted using 22C11 monoclonal antibody and lanes 5–7, using anti-GID polyclonal antibody as in section 2. Lane 1 shows tTG only; lane 2, β-APP$_{770}$ only; lane 3, tTG with $CaCl_2$; lane 4, β-APP$_{770}$, tTG and $CaCl_2$; lane 5, tTG only, lane 6, β-APP$_{770}$ only; lane 7, β-APP$_{770}$, tTG and $CaCl_2$. Arrows indicate 106 kDa monomer band, 212 kDa dimer band, and 424 kDa (or larger) multimer band, respectively.

8 minutes, as shown in the inserts in Fig. 2b–d. In these experiments, we found dimers and multimers as early as 2 min of incubation. By 40 min the multimeric band appeared to be maximal, with a concomitant decrease in the β-APP monomeric (106 kDa) band. Densitometric analysis of the three bands showed that both insoluble dimer and high M_r multimers are inversely related to the loss of the soluble monomeric β-APP (Fig. 2b–d). The loss of monomer and increase in dimers of β-APP, catalyzed by tTG, is essentially complete after 40 min incubation. At 40 min, 27 ± 1% (S.E.M.) of monomer remained, while 33 ± 1% and 41 ± 2% of dimers and multimers, respectively, had been produced. Confirming the qualitative findings, the 106 kDa β-APP monomer diminishes as a function of time, while dimeric and multimeric forms increase, forming an inverse relationship between substrate and product.

4. Discussion

Increased protein cross-linking catalyzed by tTG, accompanying the transition of a cell from a mitotic to post-mitotic state [22,23], has prompted a detailed examination of the role of tTG in the nervous system. tTG activity is present in human [24] as well as in rat brain [25], rat brain synaptosomal preparations [25] and peripheral nerves [26]. More pertinent to β-APP, the recent identification of specific glutamine and lysine residues in β-amyloid peptide (residues 1–28), which participate in tTG-catalyzed homopolymerization and heteropolymerization with α_2-macroglobulin receptor [27,28], suggests possible sites for cross-linking in the precursor. The association of β-APP with the ECM has been dem-

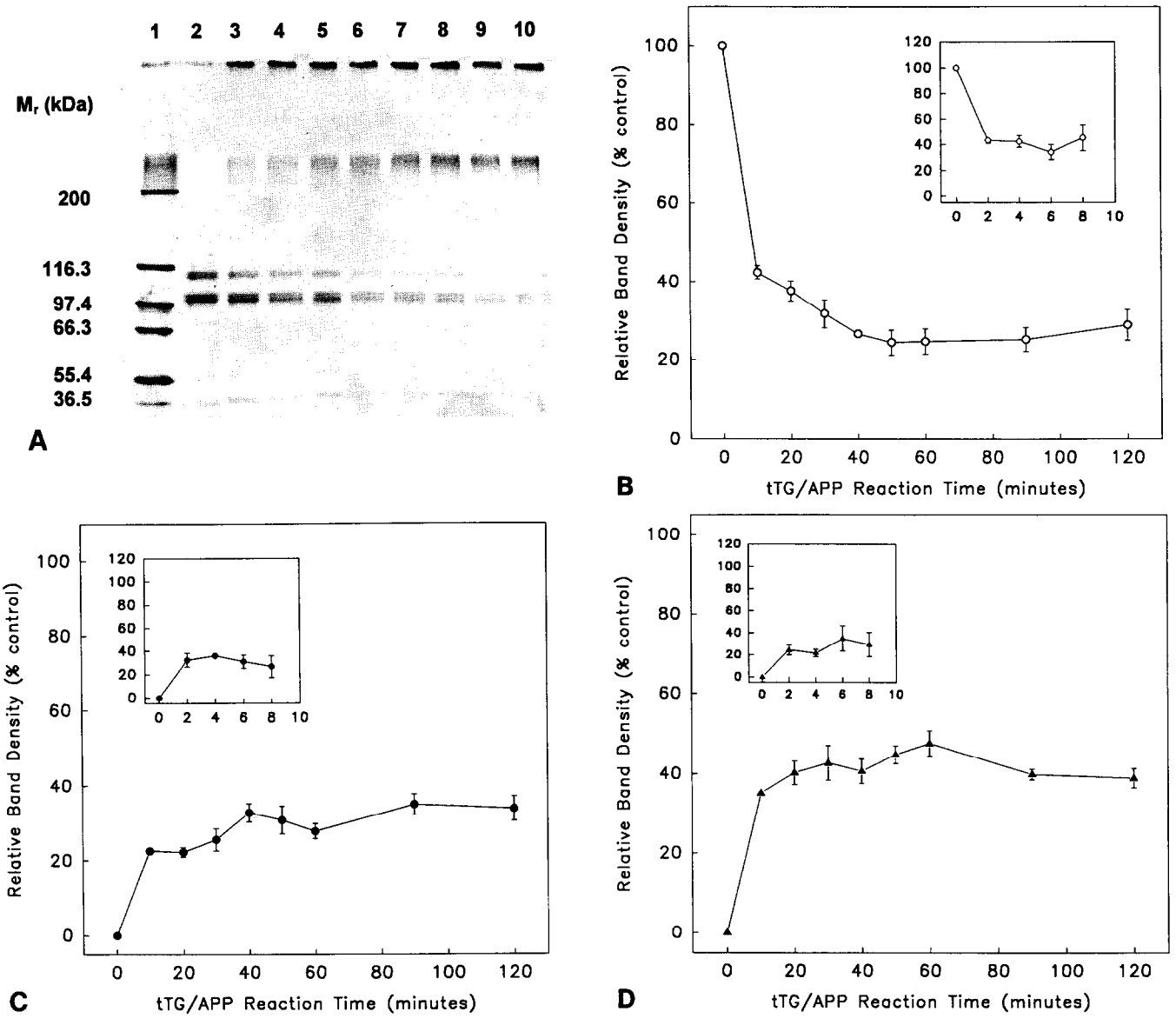

Fig. 2. Relationship between monomeric β-APP$_{770}$, dimeric and multimeric β-APP$_{770}$ over time. (a) SDS-PAGE demonstrates the amount of product and substrate over the time course. Lane 1, molecular weight markers; lane 2, time point 0; lane 3, 10 min; lane 4, 20 min; lane 5, 30 min; lane 6, 40 min; lane 7, 50 min; lane 8, 60 min; lane 9, 90 min; lane 10, 120 min. (b–d) shows densitometric analysis of the gel in 2a. Open circles are monomers, closed circles, dimers, and closed triangles, multimers. Values are expressed as relative band density as % of control, where 100% represents the band density of 60 ng of standard β-APP$_{770}$. Inserts show the course of the reaction between 0 and 10 min.

onstrated by electron microscopy [15] and accumulated evidence supports a role for β-APP in cell adhesion. Anti-β-APP antibodies prevent the adhesion of cultured cells to cell-substrata as well as neuron-neuron and neuron-glial contact in culture [15,16]. Our finding that β-APP$_{770}$ is cross-linked by tTG, reinforces the concept that the secreted protein is, or becomes, an essential component of the ECM, stabilizing cellular attachments and preventing further neuritic growth. Cross-linked β-APP, either homo- or hetero-forms, may act as a supporting infra-structure, stabilizing synaptic sites. Furthermore, cross-linking of β-APP, as a normal biochemical event for cell growth and survival, may alter, retard, or even preclude proteolytic cleavage of the precusor molecule and prevent generation of β-amyloid peptide.

The secreted forms of β-APP are identical to protease nexin II (PNII), a potent Kunitz-type inhibitor of several serine proteases including trypsin, chymotrypsin and both factors IXa and XIa of the coagulation cascade [29,30]. Since, β-APP undergoes fast anterograde transport in the axon to presynaptic sites [31], and is localized to both central and peripheral synapses [32,33], it is highly possible that the protein also becomes an essential component in the synaptic cleft ECM. Loss of synapses

correlates best with dementia [34], making defects in synaptic maintenance a strong candidate underlying Alzheimer's disease. Stable, homo- or hetero-cross-linked β-APP, from pre-synaptic to post-synaptic membrane or from synapse to ECM (or its equivalent in the brain) would also serve to stabilize potent serine protease inhibitor (i.e. PNII) activity at synaptic sites. In this regard, four other serine protease inhibitors, of some 50 known macromolecules identified, have so far been localized to vertebrate synapses. The first to be discovered was protease nexin I (PNI), a serpin related to PNII, but, in addition, α_1-antichymotrypsin, and the synapse-organizing, acetylcholine receptor-aggregating molecule, agrin, are also synapse-localized [35–37]. The presence of tTG activity at the peripheral, cholinergic, nicotinic, neuromuscular synapse [38] argues for its role at central synapses, as well. With both enzyme (tTG) and substrates, such as laminin, thrombospondin, fibronectin and, now, β-APP, significant implications for the involvement of tTG in Alzheimer's disease, where mutation or abnormal function of the enzyme may result in synapse loss, are possible.

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