Residues in the synuclein consensus motif of the α-synuclein fragment, NAC, participate in transglutaminase-catalysed cross-linking to Alzheimer-disease amyloid βA4 peptide

Poul H. JENSEN,*‡ Esben S. SØRENSEN,† Torben E. PETERSEN,† Jørgen GLIEMANN* and Lone K. RASMUSSEN†

*Department of Medical Biochemistry, University of Aarhus, DK-8000 Aarhus C, Denmark, and †Protein Chemistry Laboratory, University of Aarhus, The Science Park, DK-8000 Aarhus C, Denmark

The widespread deposition of amyloid plaques is one of the hallmarks of Alzheimer disease (AD). A recently described component of amyloid plaques is the 35-residue peptide, non-Aβ component of AD amyloid, which is derived from a larger intracellular neuronal constituent, α-synuclein. We demonstrate that transglutaminase catalyses the formation of the covalent non-Aβ component of AD amyloid polymers *in vitro* as well as polymers with β-amyloid peptide, the major constituent of AD plaques. The transglutaminase-reactive amino acid residues in the non-Aβ component of AD amyloid were identified as Gln^{79} and Lys^{80}. Lys^{80} is localized in a consensus motif Lys-Thr-Lys-Glu-Gly-Val, which is conserved in the synuclein gene family. Thus transglutaminase might be involved in the formation of insoluble amyloid deposits and participate in the modification of other members of the synuclein family.

INTRODUCTION

The characteristic neuropathological features of Alzheimer disease (AD) are the development of abundant amyloid-containing senile plaques and neurofibrillary tangles. The principal protein component of AD amyloid is a 39–42-residue peptide, βA4, whereas the microtubule-associated protein tau in a hyperphosphorylated state is the main constituent of intracellular neurofibrillary tangles. βA4 is proteolytically derived from a larger transmembrane glycoprotein, the amyloid precursor protein (APP) (reviewed in [1]).

Another peptide component, designated non-Aβ component of AD amyloid (NAC), has been discovered in a proteolytic digest of purified amyloid. Molecular cloning revealed that NAC is derived from a larger precursor protein, NACP [2], thus resembling the processing of βA4 from APP. Recently, two human synucleins, α and β, have been identified in brain tissue [3]. The α-synuclein, a 140-residue protein, was shown to be identical with NACP, which in turn is homologous to synuclein in rat brain [4] and electro-plaques of *Torpedo californa* [5]. Furthermore, human β-synuclein is homologous to bovine phosphoneuroprotein 14 [6]. The synuclein family members are characterized by a high degree of amino acid sequence similarity, particularly in the N-terminal part containing several repeats with the consensus sequence Lys-Thr-Lys-Glu-Gly-Val. Synucleins are present in a pattern that mirrors AD pathology, e.g. neuronal cell bodies and nerve terminals of the entorhinal cortex and hippocampus [2,3]. This led to the hypothesis that synucleins/NACP provide a link between the degeneration of cholinergic nerve terminals and the formation of insoluble plaques [7].

Transglutaminases (TGs) represent a family of widely distributed Ca^{2+}-dependent enzymes, which catalyse cross-linking of proteins via reactive glutamine and lysine residues (reviewed in [8]). The activity of the intracellular tissue TG has been detected in normal and AD brains, and it has been proposed that TG is involved in the development of abnormal insoluble neurofilaments [9]. Recent studies have demonstrated that tau [10], APP [11] and βA4 [12–14] are substrates for TG. Furthermore, it was shown that Gln^{15}, Lys^{16} and Lys^{28}, in βA4 are the reactive acceptor and donor sites responsible for the TG-catalysed formation of polymers [13].

Here we demonstrate that the NAC fragment of human α-synuclein forms homopolymers as well as heteropolymers with βA4 in TG-catalysed reactions. Furthermore, by the use of site-specific probes, the TG-reactive Gln^{79} and Lys^{80} residues were localized. The TG-reactive lysine was localized in one of the synuclein consensus sequences, with potential implications for the other members of the synuclein family.

EXPERIMENTAL

Materials

Amyloid NAC peptide corresponding to residues 61–95 in the NACP/α-synuclein sequence, with an extra N-terminal tyrosine residue attached in order to permit iodination (Figure 3), the dansylated peptide (dansyl-Pro-Gly-Gly-Gln-Gln-Ile-Val) (Dns-peptide) and the 40-residue βA4 mutant ($Glu^{22} \rightarrow Gln$) were synthesized at Kem-En-Tec, Copenhagen, Denmark. Amyloid β-peptide (βA4) (residues 1–40) was purchased from Bachem, Bubendorf, Switzerland. The NAC peptide stock was handled in 35% acetonitrile/0.1% trifluoroacetic acid. Before the experiments, the NAC peptide stock was diluted with Buffer A (50 mM Tris, pH 8.5, 0.5 mM dithioerythritol, 5 mM $CaCl_2$, 0.01% Triton X-100). The organic solvent was removed by freeze-drying, followed by reconstitution with water. The detergent in Buffer A was essential for the successful solubilization of the freeze-dried NAC peptide.

The NAC and βA4 peptides were iodinated, as previously described for βA4, to specific radioactivities of about 20 mCi/mg [13]. Guinea-pig liver TG and pepsin were from Sigma and Worthington, U.S.A., respectively. [1,4-^{14}C]Putrescine (109 mCi/mmol) and ^{125}I (2.0 Ci/μmol) were from Amersham

Abbreviations used: AD, Alzheimer's disease; APP, amyloid precursor protein; βA4, β-amyloid peptide; Dns-peptide, dansyl conjugated to Pro-Gly-Gly-Gln-Gln-Ile-Val; NAC, non-Aβ component of AD amyloid; NACP, NAC precursor; PTH, phenylthiohydantoin; TG, transglutaminase.

‡ To whom correspondence should be addressed.

International, U.K. The low-molecular-mass markers were from Boehringer, Mannheim, Germany and the prestained markers from Novex, San Diego, CA, U.S.A.

TG-mediated polymerization of NAC

All polymerization experiments were carried out in Buffer A with ^{125}I-labelled NAC (5×10^6 c.p.m./ml) as tracer in the presence of 50 nM guinea-pig liver tissue TG. TG activity was inhibited by 5 mM EDTA. Incubations were terminated by denaturation in reducing SDS-sample buffer, at 95 °C for 5 min, before analysis by 8–16%-gradient Tris/glycine SDS/PAGE [15] followed by autoradiography.

Labelling of NAC with site-specific probes

In the glutamine-labelling experiment, 5 μM NAC (18 μg/ml) was labelled with 100 μM [^{14}C]putrescine by 40 nM guinea-pig liver TG in Buffer A at 37 °C for 24 h. The lysine labelling was performed at 37 °C for 24 h in Buffer A, with 400 μM Dns-peptide, 16 μM NAC (58 μg/ml), and 80 nM guinea-pig liver TG. The reactions were stopped by precipitation of the proteins in 20% trichloroacetic acid. The precipitates were washed with acetone to remove unincorporated radiolabelled putrescine or Dns-peptide. The labelled NACs were analysed by SDS/PAGE 16% Tris/Tricine gels [16], photographed under UV light and stained with Coomassie Brilliant Blue, followed by autoradiography.

Purification and characterization of [^{14}C]putrescine-labelled NAC peptides

[^{14}C]Putrescine-labelled NAC was subjected to pepsin digestion at an enzyme:substrate ratio of 1:50 (w/w) in 5% formic acid at 37 °C for 2 h. Radiolabelled peptides were purified as previously described [13].

RESULTS

Transglutaminase-catalysed cross-linking of NAC to βA4

Figure 1 demonstrates the TG-catalysed polymerization of ^{125}I-NAC after 1 h of incubation. The homopolymerization was dose-dependent, as evident from the appearance of polymerization products with apparent sizes of 6 and 12 kDa with increasing NAC concentrations from 5 nM (lane 2) to 850 nM (lane 4). A similar augmentation of the polymerization of 5 nM ^{125}I-NAC was observed when 850 nM βA4 was present (lane 6), indicating the formation of covalent NAC–βA4 complexes. All polymerizations were inhibited by EDTA quenching of Ca^{2+} required for activation of TG (lanes 3, 5, 7). Whereas the ^{125}I-NAC–βA4 complexes readily formed hetero-tetramers, the NAC homopolymers primarily consisted of dimers. The high-molecular-mass radiolabelled polymers, of approx. 100 kDa and at the interphase between the stacking and resolving gels, were formed with impurities in the guinea-pig liver TG preparation, as they also appeared when other peptides were polymerized with the TG preparation (results not shown).

TG-reactive acceptor and donor sites in NAC

Radiolabelled putrescine and the fluorescent dansylated peptide were used as site-specific probes for labelling of TG-reactive glutamine and lysine residues, respectively [17,18]. As shown by SDS/PAGE (Figure 2), TG catalysed both the incorporation of [^{14}C]putrescine (lane 3) as well as that of the fluorescent Dns-peptide (lane 4). The labelling by both probes was completely abolished by the presence of EDTA (results not shown).

As NAC only contains one lysine residue, Lys^{80}, in the NACP/α-synuclein sequence [2], this represents the TG donor site. In order to identify which of the glutamine residues, Gln^{62} and/or Gln^{79}, was the TG acceptor site, NAC was labelled with [^{14}C]putrescine, digested with pepsin, and the resulting peptides were separated by reverse-phase HPLC (Figure 3a). Samples from each peak were assayed for radioactivity by liquid-scintillation counting. Five peaks, designated P1–P5, contained

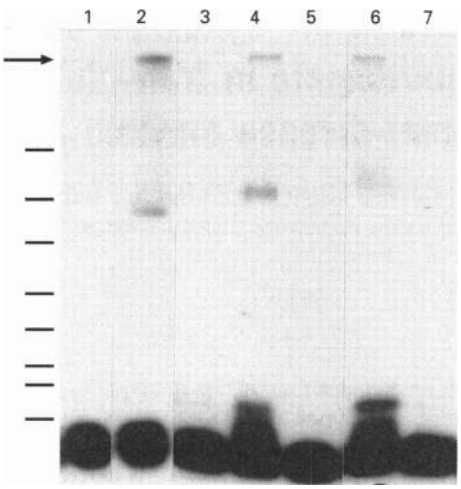

Figure 1 TG-catalysed homopolymerization of NAC and hetero-polymerization with βA4

^{125}I-NAC (5 nM; lane 1) was incubated with 50 nM guinea-pig liver TG for 1 h (lane 2) in the presence of 850 nM unlabelled NAC (lane 4) or βA4 (lane 6). Lanes 3, 5 and 7 are like lanes 2, 4 and 6, but contain 5 mM EDTA. All samples were processed for reducing 8–16%-gradient SDS/PAGE. The autoradiogram is presented with the positions of the prestained molecular-mass markers (kDa) shown to the left: 250, myosin; 98, BSA; 64, glutamate dehydrogenase; 50, alcohol dehydrogenase; 36, carbonic anhydrase; 30, myoglobulin; 16, lysozyme; 6.0, aprotinin. The abnormal sizes of the marker proteins are due to the prestaining. The arrow marks the interphase between the stacking gel and the resolving gel.

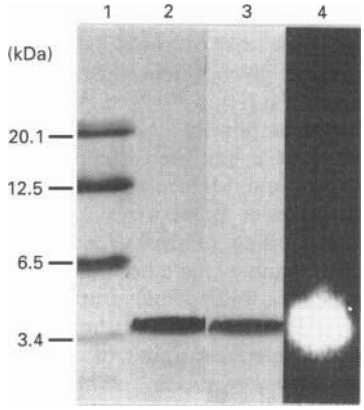

Figure 2 Labelling of TG-reactive glutamine and lysine residues in NAC

NAC peptide was labelled with [^{14}C]putrescine or Dns-peptide by guinea-pig liver TG for 24 h at 37 °C. The peptide was resolved by SDS/PAGE. Lane 2 shows the Coomassie-Blue-stained peptide. Lane 3 demonstrates fluorography of the [^{14}C]putrescine-labelled peptide, and lane 4 presents the fluorescence of the Dns-peptide-labelled NAC under UV light. Molecular-mass markers (kDa) are shown in lane 1.

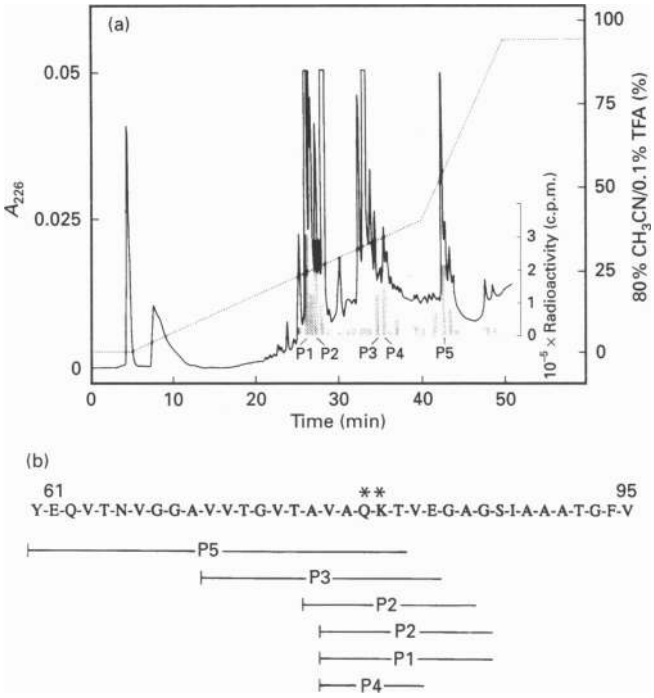

Figure 3 Isolation and characterization of [^{14}C]putrescine-labelled NAC peptides generated by pepsin digestion

(**a**) [^{14}C]Putrescine-labelled NAC digested with pepsin. The resulting peptides were separated on a Vydac C_{18} (10 μm) reverse-phase HPLC column using 0.1% trifluoroacetic acid (TFA) (solvent A) and 80% acetonitrile/0.1% TFA (solvent B) with a stepwise linear gradient (dotted line). The flow rate was 0.5 ml/min and the column temperature was 40 °C. Peptides were detected in the effluent by recording the A_{226} (continuous line) and collected manually. Shaded bars indicate total amount of radioactivity in the respective peaks as determined by liquid-scintillation counting. (**b**) N-terminal amino acid sequence analysis of the isolated [^{14}C]putrescine-labelled peptides. The amino acid sequence of the investigated NAC peptide is shown, with the TG-reactive glutamine and lysine residues marked by asterisks. Characterized peptides found in peaks P1–P5 are indicated by bars. Amino acids are numbered according to α-synuclein/NACP.

significant amounts of radioactivity. The amino acid sequence data from the peptides in these peaks are summarized in Figure 3(b). Sequence analyses of P1–P5, containing Gln^{79}, all showed a low yield of PTH-Gln (PTH, phenylthiohydantoin) in the corresponding cycles, indicating a putrescine-linked glutamine residue. Scintillation counting of the PTH-amino acids confirmed the presence of radioactivity only in these cycles. In contrast, no labelling was found at Gln^{62}, present in P5. This shows conclusively that the NAC fragment harbours both TG donor and acceptor sites capable of participating in cross-linking reactions forming homopolymers and heteropolymers with βA4.

DISCUSSION

NAC was recently identified as two peptides in a *Achromobacter lyticus* proteinase I digest of purified AD amyloid [2]. Subsequent cDNA cloning from a human fetal brain library revealed that the two NAC peptides are encoded in tandem in a 19 kDa protein, NACP [2]. The N-terminus of NAC and the cleavage site between the two peptides (Lys^{80}–Thr^{81}) agree with the proteinase specificity of *Achromobacter lyticus* proteinase I which cleaves C-terminally to lysine residues [19]. However, the C-terminal of the NAC peptide is not generated by this proteinase. Thus, NAC used in this study represents the peptide purified from amyloid

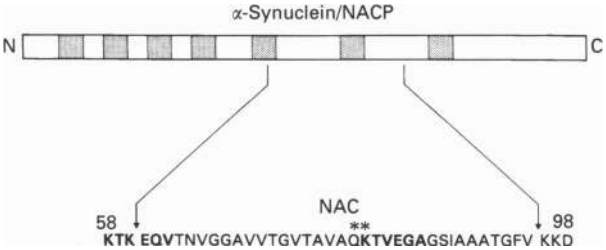

Figure 4 Schematic model of α-synuclein/NACP showing the structure of the NAC peptide region

The characteristic repetitive motifs containing the consensus sequence, KTKEGV, are shown as shaded boxes. TG-reactive lysine and glutamine residues in NAC are marked by asterisks, and the motifs are shown in bold. Amino acids are numbered according to α-synuclein/NACP.

and might represent the C-terminal part of a larger NACP-derived peptide present in AD amyloid.

A unique structural feature of NACP is the presence of seven repeated sequence motifs (Figure 4) with the consensus sequence Lys-Thr-Lys-Glu-Gly-Val [2]. The repetition of this motif is a conserved feature of the synuclein family, present in fish [5], rats [4], cows [6] and man [3]. NACP is identical with human α-synuclein [3]. The synucleins are present in neural cell bodies and presynaptic nerve terminals. The expression of α-synuclein/NACP in areas of the brain mirroring the distribution of AD pathology [2,3] formed the basis for the hypothesis that α-synuclein/NACP represents a link between the degeneration of cholinergic nerve terminals and AD plaques [7].

We demonstrate that NAC is a substrate for TG and identify Gln^{79} and Lys^{80}, but not Gln^{62}, as the TG-reactive amino acids. Still the lack of labelling of Gln^{62} might be an artefact, due to the lack of the original sequence preceding the Gln^{62} residue (Figure 4). It is noteworthy that the TG-reactive Lys^{80} is situated in the sixth consensus motif in α-synuclein/NACP (residues 80–85). Whether all lysine residues in the synuclein motifs are TG-reactive is still unknown. The Gln^{79}, being outside the synuclein motif, is not conserved among the synucleins, but several of the motifs contains glutamine residues, e.g. motifs 2 and 5 in human α-synuclein/NACP [2].

Whereas βA4 readily forms multimers up to pentamers [13], NAC preferably forms dimers. The structural basis for this difference is probably the presence of two TG-reactive lysine residues in βA4, of which one is situated 13 residues apart from the reactive glutamine. In contrast, the investigated NAC peptide contains only one TG-reactive lysine and one glutamine residue. The formation of NAC polymers larger than dimers requires the participation of both residues on the same peptide, which appears to be sterically unfavourable, since they are next to each other.

Dutch-type amyloidosis, characterized by distinct amyloid depositions around vessels, is caused by a point mutation in the APP gene changing Glu^{22} to a glutamine residue in the βA4 sequence. This mutant was recently demonstrated as a TG substrate, which could form polymers like the wild-type βA4 [14] and NAC (results not shown). We raised the question whether the additional Gln^{22} in the βA4 mutant peptide could participate in cross-linking reactions. Analysis of the mutant βA4 peptide (a synthetic 40-residue peptide), containing two glutamine residues, showed that the additional Gln^{22} was not able to incorporate putrescine, thus not being a TG-acceptor site (results not shown). Still, comparison of the solution structure of βA4-(1–28) wild-type and Dutch-type reveals a different conformation [20]. Thus

different TG-substrate properties could be due to a different presentation of the reactive residues.

The role of TG in the central nervous system has not yet been determined. However, TG activity compatible with the tissue type of TG has been detected in normal and AD brains [9]. Moreover, rat brain slices subjected to tetanic stimuli contained increased levels of the TG activity product, the Gln-Lys isopeptide [21]. In this context it is of interest that TG has been implicated in participating in cellular signalling by the modulation of growth factor/cytokine activity, e.g. interleukin-2 [22] and transforming growth factor β [23]. Finally, the recent cloning of a novel G-protein in rat revealed that the protein was a functional tissue TG acting as a G-protein in rat liver [24]. These findings emphasize that TG could be involved in several functions in the central nervous system. Pertinent to AD, TG might participate in the metabolism of APP [11], βA4 [12,13], tau [10] and the α-synuclein fragment NAC, since they all have been shown to be TG substrates. Hypothetically, conformationally changed TG-dimerized βA4 or NAC molecules might serve as seeds that promote the polymerization of monomeric βA4, as demonstrated for anti-chymotrypsin and lipoprotein E4 [25].

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