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Inhibition of trypsin by the β-amyloid protein precursor

A comparative study between transfected cells, human brain and cerebrospinal fluid

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Soluble β-amyloid protein precursors (β-APPs) were studied in human brain and cerebrospinal fluid (CSF) after partial purification by ion exchange chromatography. Proteins were analysed in immunoblotting experiments using a monoclonal antibody directed against the N-terminal segment of the β-APP 770, and by reverse enzymography. In the human brain and CSF, a protein which comigrates with the β-APP 770 expressed by transfected CHO cells was able to inhibit trypsin.

β-Amyloid precursor; Charge separation; Inhibition of serine protease

1. INTRODUCTION

Alzheimer's disease (AD) is a neurodegenerative disorder affecting aged people. The senile plaques are with neurofibrillary tangles, characteristic neuropathological features of AD. A peptide of 39–42 amino acids called A4 or β-amyloid peptide has been identified as a major constituent of the amyloid deposits observed in senile plaques and in the cerebrovascular angiopathy [1,2]. The β-amyloid peptide was observed to be part of a large precursor containing 695 amino acids (β-APP 695) which resembles a transmembrane protein [3]. Several other β-APP isoforms have been identified including β-APP 751, β-APP 770 and β-APP 563 [4–7]. They differ from β-APP 695 by the presence of additional sequences in the N-terminal domain which are 50% homologous with the Kunitz-type serine protease inhibitors [4–6]. In addition, the β-APP 563 lacks the C-terminal domain, including the transmembrane region, present in other β-APPs. Soluble β-APPs found in cell culture medium, human brain extracts or CSF [8] are obtained after a proteolytic cleavage of transmembrane β-APPs. This cleavage occurs inside the β-amyloid peptide [9].

In AD, an increase of the expression of β-APP isoforms showing a protease inhibitory activity [5,10–13] might be related to the formation of amyloid deposits, by impending the activity of proteases normally involved in the metabolism of β-APP. This is further suggested by the fact that amyloid deposits have been reported to occur in transgenic mice carrying a β-APP 751 gene [14]. Amyloid deposits were also found in transgenic mice overexpressing the β-amyloid peptide [15].

Cultured cells expressing the β-APP isoforms containing the Kunitz inhibitor domain were previously reported to inhibit trypsin, a well-known serine protease [6,16]. In this study, we have investigated the inhibition of trypsin by soluble β-APPs found in human brain extracts and CSF in comparison to that found in the culture medium of transfected cells.

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2. MATERIALS AND METHODS

The β-APP 770-pKCR3 expression shuttle and a pSV_2NEO neomycin resistance gene vector were cotransfected into CHO cells using the calcium phosphate precipitation method [17]. On day 2 after transfection, transfected cells were selected by growing them in the presence of 600 μg/ml of geneticin (G418). Expression of β-APP 770 was measured in immunoblotting experiments using a monoclonal antibody raised against the N-terminal domain of β-APP 770. This mouse IgA monoclonal antibody was obtained after inoculation of a bacterial fusion protein between β-galactosidase and a fragment of APP 770 encompassing the sequence between amino acids 81 and 481. Having established a CHO cell population expressing the β-APP 770, this mixed population was subjected to limiting dilution to produce individual clonal cell lines. The monoclonal cells were then cultured for 24 h in Ham F12 medium (Gibco) without foetal calf serum. This culture medium, containing secreted β-APP 770, was desalted by Sephadex G25 chromatography and concentrated by lyophylisation prior to SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

One gram of cerebral frontal cortex isolated from normal human brain was homogeneized using a Dounce homogenizer, in 4 ml of 50 mM Tris-HCl (pH 7.6), 1 mM EDTA, 1 μg/ml leupeptin, 5 μg/ml aprotinin (buffer A) and centrifuged for one hour at 50,000 × g. The supernatant of the brain homogenate was further analysed. CSF from non-AD patients was precipitated by 60% $(NH_4)_2SO_4$ and centrifuged for 30 min at 10,000 × g. The pellet was resuspended and dialysed against buffer A. The soluble fraction of the brain homogenate and CSF were applied onto DEAE-cellulose column (Whatman) previously equilibrated in buffer A. Proteins were eluted by buffer A con-

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taining 150 mM, 300 mM or 600 mM NaCl. The fraction of CSF eluted by 0.3 M NaCl was further analyzed by HPLC chromatography on Mono-Q column (Pharmacia) as described by Palmert et al. [18]. All samples were desalted and concentrated prior to SDS-PAGE.

Proteins were electrophoresed on 7.5% polyacrylamide gels in Laemmli buffer and were transferred to nitrocellulose [19]. After a blocking step in 5% non-fat dry-milk, the nitrocellulose was incubated with the specific anti-APP monoclonal antibody, a biotinylated goat anti-mouse IgA (Sigma) and developed by the streptavidin-alkaline phosphatase conjugated system (Boehringer). For reverse enzymography [20], gels were run in non-denaturating conditions (i.e. in the absence of β-mercaptoethanol and without boiling step). After electrophoresis, gels were washed at room temperature in 100 mM Tris-HCl (pH 8.1), 2.5% Triton X-100 and finally in 100 mM Tris-HCl. The gels were then layered on 0.8% agar gel containing 15 mg/ml non-fat dry-milk solubilized in 100 mM Tris-HCl (pH 8.1) and 50 ng/ml trypsin (Sigma) and incubated for 16 h at 37°C. Trypsin was able to digest and clarify casein but not where trypsin inhibition occurred.

3. RESULTS

The inhibition of trypsin by soluble β-APPs found in human brain and CSF was compared to that of secreted β-APP 770 expressed by transfected CHO cells. A 50,000 × g supernatant of a human brain homogenate and total CSF were analysed in immunoblotting experiments together with the soluble form of the β-APP 770 found in the culture medium of transfected CHO cells. Fig. 1A shows that two proteic bands (●,▲) were specifically recognized by the anti-APP monoclonal antibody in brain and CSF. The upper band comigrates with the β-APP 770 found in the culture medium of CHO cells transfected with the corresponding cDNA sequence. Additional bands (lane 2) were also detected when the first specific monoclonal antibody was not added (lane 4), and therefore are due to the revelation system used. Fig. 1B shows the analysis of the same samples by reverse enzymography, allowing one to measure the ability of a protein to inhibit the trypsin-induced digestion of casein. β-APP 770 found in the culture medium of transfected CHO cells is able to inhibit trypsin, as it contains the Kunitz protease inhibitor domain [13]. In

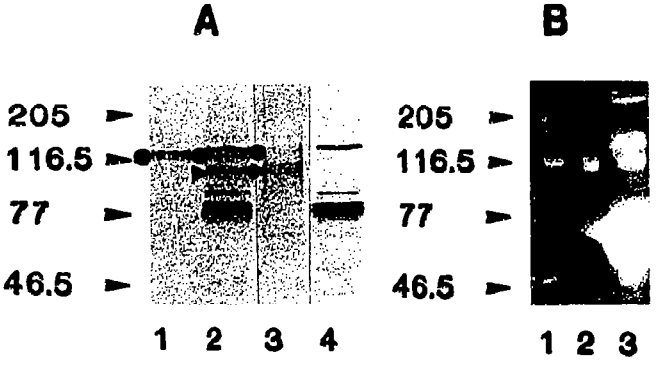

Fig. 1. Western blot analysis after SDS-PAGE (A) or reverse enzymography after electrophoresis in non-denaturating conditions (B). (Lane 1) Culture medium from β-APP 770 CHO transfected cells. (Lane 2) Soluble fraction of brain homogenate. (Lane 3) CSF. (Lane 4) Soluble fraction of brain homogenate revealed without the first specific monoclonal antibody.

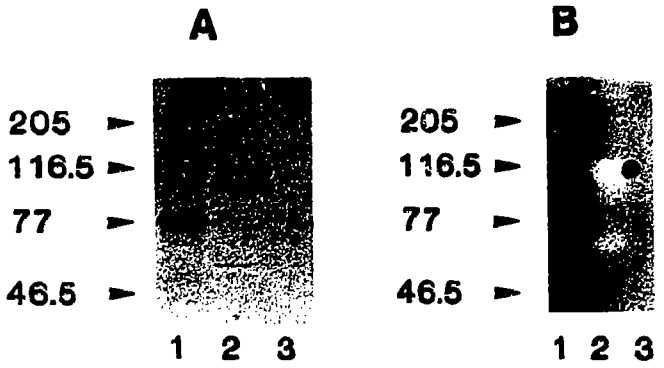

Fig. 2. Purification of β-APPs from the soluble fraction of brain homogenate, using DEAE chromatography. (A) Western blot analysis. (B) Reverse enzymography. (Lane 1) 0.15 M NaCl eluted proteins. (Lane 2) 0.30 M NaCl eluted proteins. (Lane 3) 0.60 M NaCl eluted proteins.

the human brain, an inhibitory activity (●) comigrates with that found in the culture medium of transfected cells. While the situation in CSF is far more complex, an inhibitory activity is also detected at the same molecular weight.

In order to clarify the results, we decided to purify the proteins recognized by the monoclonal antibody using anionic chromatography on DEAE-cellulose and a Mono-Q HPLC column. Fig. 2 shows that the ion exchange chromatography was able to dissociate the specific immunolabelling from the non-specific one. Fig. 2A shows that the doublet specifically recognized by the anti-APP monoclonal antibody (●,▲) is eluted at 0.3 M NaCl. The inhibitory activity (●) is recovered in the same fraction at a molecular weight which could correspond to the high molecular form of the two proteins recognized by the monoclonal antibody (Fig. 2B).

The human CSF was submitted to the same analysis after ammonium sulfate precipitation (Fig. 3). As for human brain, the doublet recognized by the antibody was eluted from the DEAE column in the fraction containing 0.3 M NaCl (Fig. 3A, lane 3). In reverse enzymography, many different inhibitory activities were detected in the same fraction. It is the reason why we further rechromatographed this 0.3 M fraction on a HPLC Mono-Q column. At 0.6 M NaCl concentration, the proteic bands recognized by the antibody were eluted (Fig. 3A, lane 4). In reverse enzymography, this fraction was shown to exhibit a trypsin inhibitory activity concentrated in two proteins (Fig. 3B, lane 4). A strong inhibition of trypsin was detected at a molecular weight corresponding to the high molecular weight β-APP isoform recognized by the anti-APP monoclonal antibody. A weak inhibitory signal was also detected in a region of the gel where the second APP isoform is found. In the total CSF, however, this inhibitory activity was not detected (Fig. 3B, lane 1), in spite of the recognition of the low molecular weight APP isoform

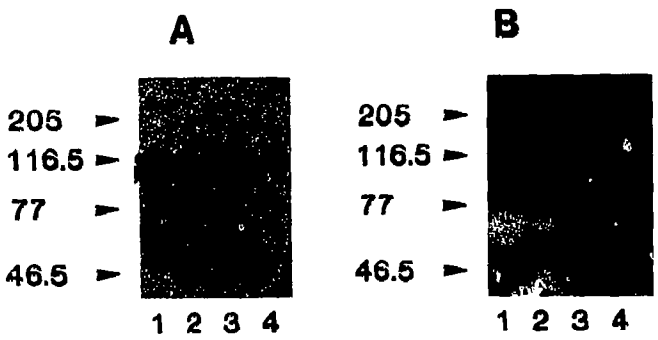

Fig. 3. Purification of β-APPs from CSF by DEAE- and Mono-Q-chromatography. (A) Western blot analysis. (B) Reverse enzymography. (Lane 1) Total CSF. (Lane 2) $(NH_4)_2SO_4$ precipitated CSF. (Lane 3) 0.3 M eluted fraction. (Lane 4) Mono-Q eluted fraction.

by the specific monoclonal antibody (Fig. 3A, lane 1). Therefore, this inhibition of trypsin seems unrelated to APP.

4. DISCUSSION

Molecular cloning allowed the identification of β-APP isoforms containing sequences in their extracellular domain which show homology with serine protease inhibitors of the Kunitz family. Using chromogenic substrates, transfected cells expressing the β-APP 770 were shown to exhibit a trypsin inhibitory activity [6]. The protease inhibitory properties of the β-APP 751 expressed as a bacterial fusion product were also demonstrated by protease inhibition assays using chromogenic substrates [21]. While very sensitive, these assays do not identify the proteins carrying the inhibitory activity. The reverse enzymography is a protease inhibition assay which allows the identification of proteins showing inhibitory activities, after their isolation by non-denaturating gel electrophoresis. Using this protease inhibition assay, we report here that a protein found both in the human brain and CSF which is recognized by an anti-APP monoclonal antibody is able to inhibit trypsin. This protein which comigrates with the β-APP 770 expressed by transfected cells most probably corresponds to a β-APP isoform containing the Kunitz protease inhibitor (KPI) domain. In the same way, an immunoblotting analysis of the human CSF using an anti- KPI antibody, showed that only the high molecular weight β-APP isoform contains the KPI domain [18].

In AD, an increase of the expression of β-APP isoforms showing a protease inhibitory activity, as reported in several studies [10–13,22], might be related to the formation of amyloid deposits, since amyloid deposits have been reported to occur in transgenic mice carrying a β-APP 751 gene [14]. While the protease specificity of the KPI domain has been investigated in vitro [23], the identification of the physiological substrate of the cerebral APP isoforms containing the KPI domain awaits further investigations.

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