Molecular Studies Define the Primary Structure of α1-Antichymotrypsin (ACT) Protease Inhibitor in Alzheimer’s Disease Brains

**COMPARISON OF ACT IN HIPPOCAMPUS AND LIVER**

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An α1-antichymotrypsin-like serpin has been implicated in Alzheimer’s disease (AD) based on immunohistochemical detection of α1-antichymotrypsin (ACT) in amyloid plaques from the hippocampus of AD brains. The presence of neuroendocrine isoforms of ACTs and reported variations in human liver ACT cDNA sequences raise the question of the molecular identity of ACT in brain. In this study, direct reverse transcription-polymerase chain reaction and cDNA sequencing indicate that the hippocampus ACT possesses the reactive site loop that is characteristic of serpins, with Leu as the predicted P1 residue interacting with putative chymotrypsin-like target proteases. The deduced primary sequence of the human hippocampus ACT possesses more than 90% homology with reported primary sequences for the human liver ACT. Moreover, identical ACT primary sequences deduced from the cDNAs were demonstrated in the hippocampus of control and AD brains. Northern blots showed that ACT mRNA expression in hippocampus was 900 times lower than that in liver. Also, hippocampus and liver ACT proteins demonstrated differential sensitivities to deglycosylation. Overall, reverse transcription-polymerase chain reaction combined with cDNA and primary sequence analyses have defined the molecular identity of human hippocampus ACT in control and AD brains. The determined reactive site loop domain of hippocampus ACT will allow prediction of potential target proteases inhibited by ACT in AD.

A role for the protease inhibitor α1-antichymotrypsin in Alzheimer’s disease (AD) has been suggested based on immunohistochemical detection of ACT in amyloid plaques in brains of AD patients (1, 2).

ACT is a member of the serine protease inhibitor family, known as serpins, which typically possesses a reactive site loop (RSL) domain that interacts with target proteases (9, 10). Recently, molecular cloning has identified isoforms of ACT in bovine neuroendocrine tissues of adrenal medulla and pituitary that differ in their RSL domains (3, 4). Differences in RSL predict that these ACT isoforms inhibit different target proteases; indeed, expression of these isoforms have demonstrated the protease-specific nature of these ACT isoforms.2 Furthermore, variations in the deduced primary sequences of human liver ACT cDNAs have been reported (5–8). Because these ACT isoforms are all recognized by anti-ACT sera, these observations raise the question of the molecular identity of ACT-like immunoreactivity in Alzheimer’s disease brains. However, the primary structure of ACT encoded by human brain ACT cDNA has not been elucidated.

Therefore, the goal of this study was to determine the molecular identity of the ACT cDNA expressed in AD and normal hippocampus, a brain region abundant in amyloid plaques in AD, as well as to characterize the brain ACT. Direct reverse transcriptase polymerase chain reaction (RT-PCR) and DNA sequence analyses have defined the primary sequence of ACT in Alzheimer’s and normal brains. Moreover, the defined primary sequence of the human hippocampus ACT cDNA sequence resolves its identity compared with reported variations in human liver ACT cDNA sequences (5–8). Primary sequence comparisons indicate that the human hippocampus and liver ACTs resemble one another with greater than 90% homology. Further analyses of hippocampus and liver ACTs were performed with respect to transcription initiation sites and expression of the ACT gene as well as the glycoprotein nature of ACT. This study has, thus, defined the primary sequence and characteristics of ACT expressed in control and Alzheimer’s disease brains.

**EXPERIMENTAL PROCEDURES**

RT-PCR and DNA Sequencing of ACT cDNAs from Hippocampus of Alzheimer’s Disease and Normal Brains—To obtain the segment of the hippocampus ACT cDNA corresponding to the predicted open reading frame (ORF) encoding the primary sequence of the hippocampus ACT, RT-PCR and DNA sequence analysis of overlapping 790- and 502-bp 5′ and 3′ cDNA fragments, respectively, of human hippocampus ACT cDNA from AD and normal brains was performed (see Fig. 1). In addition, RT-PCR also generated a 296-bp DNA fragment that represents the 3′-untranslated region (UTR) that overlaps with the 502-bp cDNA fragment. PCR primers were designed based on reported human liver ACT cDNA sequences (5–8). RT-PCR of poly(A) RNA from normal and Alzheimer’s hippocampus was conducted three times, each

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**Footnotes:**

1 The abbreviations used are: AD, Alzheimer’s disease; RSL, reactive site loop; RT-PCR, reverse transcriptase polymerase chain reaction; ORF, open reading frame; bp, base pair(s); UTR, untranslated region; SSPE, saline/sodium phosphate/EDTA; PTP, prohormone thiol protease.

2 S-R. Hwang and V. Y. H. Hook, manuscript in preparation.
time with RNA isolated from a separate sample of tissue; two to four subclones from each PCR reaction were analyzed by DNA sequencing. Total RNA was isolated from frozen hippocampus tissue from AD and normal brains with the TRIZOL™ reagent (Life Technologies, Inc.) (tissues were from the Harvard Brain Tissue Resource Center at McLean Hospital, Belmont, MA) and DNA reagents from Qiagen (Valencia, CA and U.S. Biochemical Corp., Sequence Version 2.0 sequencing kit, American Pharmacia Biotech, as described previously (3, 4). Primers for DNA sequencing utilized reverse and forward primers corresponding to vector M13 sequences that flank the DNA insert. DNA sequencing of the appropriate size (assessed by digestion of the plasmid with EcoRI) were subjected to automated DNA sequencing with fluorescent-dye-labeled nucleotide triphosphates (from the Harvard Brain Tissue Resource Center at McLean Hospital, Belmont, MA) and the First Department of Anatomy, Semmelweis University, Budapest, Hungary). Frozen tissue (100 mg aliquots) was pulverized in liquid N$_2$, solubilized in 1.0 ml of TRIZOL reagent, extracted with 0.2 ml chloroform, isomylalcohol (49/1, v/v), and incubated at room temperature for 5 min. The sample was then centrifuged at 12,000 × g at 4 °C for 15 min, and the resultant RNA in the aqueous phase was precipitated with 2.5 vol ethanol, washed with 0.1 M NaCl and 0.015 M sodium citrate, and bound poly(A)$^+$ RNA was eluted with diethyl pyrocarbonate-treated water and concentrated by ethanol precipitation. In addition, poly(A)$^+$ RNA from normal human hippocampus and liver were purchased (CLONTECH) for RT-PCR. RT-PCR of overlapping 790- and 502-bp cDNA fragments that span the 3'UTR of the highly conserved amino terminal domain of ACT was performed with SuperScript II reverse transcriptase (200 units, Life Technologies, Inc.) and PCR reagents from Promega (Madison, WI). Poly(A)$^+$ RNA in the RNA sample (100 μg) was annealed to the biotinylated oligo(dT) probe at room temperature. The oligo(dT)poly(A)$^+$ hybrid was bound to streptavidin paramagnetic particles and washed with 0.1× SSC (1× SSC, 0.15 M NaCl and 0.015 M sodium citrate), and bound poly(A)$^+$ RNA was eluted with diethyl pyrocarbonate-treated water and concentrated by ethanol precipitation. In addition, poly(A)$^+$ RNA from normal human hippocampus and liver were purchased (CLONTECH) for RT-PCR.

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**RESULTS**

**RT-PCR of Hippocampus A2c Defined the ORF Encoding the A2c Primary Sequence**—RT-PCR was used to determine the primary sequence of A2c expressed in hippocampus from AD and normal brains. Primers for RT-PCR were designed based on reported homologous sequences of neuroendocrine and liver A2cs (3–8) for amplification of the mature coding region of A2c (without the NH$_2$-terminal signal sequence) (Fig. 1). RT-PCR and DNA sequence analyses for each overlapping DNA segment were performed from three separate tissue samples of control hippocampus and from three separate samples of AD hippocampus. Poly(A)$^+$ RNA from each tissue sample was subjected to RT-PCR to amplify 3′ and 5′ regions of the A2c cDNA. From each RT-PCR reaction, DNA inserts from two to four subclones were subjected to DNA sequence analyses.

The predicted 790-bp and 502-bp DNA fragments representing 5′ and 3′ domains of the hippocampus A2c cDNA, respectively, were predicted to include the RSL and the NH$_2$-terminus of mature, processed A2c, which begins at the COOH terminus of the signal peptide sequence. Primers 5 and 6 allowed amplification of the 3′-UTR of the cDNA. Primer A was used in primer extension analyses of A2c gene transcripts.

**Deglycosylation of Hippocampus A2c—**Proteins were extracted from frozen hippocampus (approximately 4 g of tissue) from Alzheimer’s disease or normal brains (tissues were from the Harvard Brain Tissue Resource Center at McLean Hospital, Belmont, MA) by the TRIZOL$^\text{TM}$ reagent (Life Technologies, Inc.). For deglycosylation of protein extracts from hippocampus and of human liver A2c (from Athens Research and Technology Biochemicals), ACT samples were incubated with N-glycosidase F (0.2 units, Boehringer Mannheim) at 37°C for 18 h in buffer (25 µl total volume) consisting of 20 mM sodium phosphate, pH 7.2, 10 mM sodium azide, 50 mM EDTA, and 0.5% (w/v) octylglucoside. Samples were then subjected to Western blots, as described previously (11, 12), with affinity-purified anti-ACT IgGs (1:100 final dilution), detected with anti-rabbit goat IgGs conjugated to alkaline phosphatase (from Promega, performed according to the manufacturer’s procedure).

For affinity purification of anti-A2c for Western blots, IgGs (immunoglobulins) were purified from anti-A2c serum (ART Biochemicals) by protein A-Sepharose chromatography (according to the manufacturer’s protocol, Amersham Pharmacia Biotech). Anti-A2c IgGs were then affinity-purified on an A2c-Sepharose affinity column. The A2c affinity column was obtained by covalently linking A2c (from human liver, ART Biochemicals) to CNBr-activated Sepharose (Amersham Pharmacia Biotech, according to the manufacturer’s instructions). The anti-A2c IgGs were bound to the A2c affinity column in equilibration buffer consisting of 60 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.05% Tween 20. After washing with equilibration buffer, anti-A2c IgGs were eluted with elution buffer 1 (50 mM Tris-HCl, pH 7.5, 750 mM NaCl, 0.05% Tween 20), followed by elution buffer 2 (0.1 M citric acid-NaOH, pH 4.0, 750 mM NaCl, 0.05% Tween 20). Fractions containing anti-A2c IgGs were detected by binding to A2c in enzyme-linked immunosorbent assay, performed as described previously (13). Affinity-purified anti-A2c IgGs were concentrated by a Centricon-30 apparatus (Pierce).

**RT-PCR of Hippocampus A2c from Normal and AD Brains**—Fig. 2. RT-PCR of the 5′ and 3′ domains of A2c cDNA from hippocampus of AD and normal brains. A, RT-PCR of the 5′ domain of A2c cDNA, detected by DNA agarose gels. RT-PCR with primers 1 and 2 generated a 790-bp DNA band from poly(A)$^+$ RNA isolated from hippocampus (H) of AD and normal (N) brains (lanes 1 and 2, respectively) as well as from human liver (L) (lane 3). B, RT-PCR of the 3′ domain of A2c cDNA. RT-PCR with primers 3 and 4 generated a DNA band of approximately 500 bp from poly(A)$^+$ RNA isolated from hippocampus (H) of AD and normal (N) brains (lanes 1 and 2, respectively) as well as from human liver (L) (lane 3).
sequences (5–8). The hippocampus ACT differs by 37 residues from the human liver cDNA sequence reported by Chandra et al. (5) at residues 79–93, 100–105, and 398–400 as well as by 10 residues at the COOH terminus. In addition, the hippocampus ACT, compared with the liver ACT cDNA reported by Chandra et al. (5), possesses different amino acids for residues 69, 199, and 338. However, the human hippocampus ACT cDNA sequence is identical to the human liver ACT cDNA reported by Rubin et al. (6) and others (7, 8). These results have therefore, defined the primary sequence of human hippocampus ACT and have resolved its similarity to human liver ACT.

Northern Analysis—Northern blots were performed to compare ACT mRNAs in human hippocampus and liver (Fig. 4). The hippocampus and liver ACT mRNAs are similar in electrophoretic mobility corresponding to 1.5 and 1.6 kilobases, respectively; these results also show slight differences in apparent size of the ACT mRNAs from these two tissues. It is noted that no significant differences in ACT mRNA levels were detected in hippocampus from AD (n = 5) and normal (n = 8) brains.

Further studies compared the 3'-UTR region of the hippocampus ACT cDNA with that of the liver ACT cDNA. RT-PCR of hippocampus poly(A)+ RNA was performed (primers 5 and 6, Fig. 1) to generate a 296-bp DNA fragment encoding the 3'-UTR region (data not shown). DNA sequence analysis indicated that the human hippocampus ACT cDNA (shown in bold for nucleotides 111 to 1576), whose deduced primary sequence (shown in bold for residues 27 to 398) corresponds to the mature ACT protein, lacks the signal sequence. The RSL domain is boxed, with the predicted P1 residue as Leu underlined. Consensus glycosylation sites are indicated by asterisks under the Asn residues as possible sites of glycosylation. The predicted 5'-region analyzed by primer extension is shown (not bold) for nucleotides 1–110 (6, 17).

FIG. 3. Complementary DNA sequence of human hippocampus ACT. The ACT cDNAs obtained from hippocampus of normal brains is illustrated. The open reading frame domains of ACT cDNAs from normal and Alzheimer’s disease brains were identical. The DNA sequence of the 3'-UTR domain of the hippocampus ACT cDNA (from normal brain) was also determined. Alignment of the DNA sequences determined for overlapping 5' and 3' PCR fragments indicates the human hippocampus ACT cDNA (shown in bold for nucleotides 111 to 1576), whose deduced primary sequence (shown in bold for residues 79 to 398) corresponds to the mature ACT protein. The positions of primers 1–6 used in RT-PCRs are shown by dotted lines with arrows. Arrows above the His (+1) and Asn (+3) residues indicate the predicted NH2 terminus of the mature ACT, which lacks the signal sequence. The RSL domain is boxed, with the predicted P1 residue as Leu underlined. Consensus glycosylation sites are indicated by asterisks under the Asn residues as possible sites of glycosylation. The predicted 5'-region analyzed by primer extension is shown (not bold) for nucleotides 1–110 (6, 17).
cated that the 3′-UTR of the hippocampus ACT cDNA (shown in Fig. 3) is nearly identical to the reported human liver ACT cDNAs (5, 6), with greater than 98% homology in nucleotide sequence within the 296-bp 3′-UTR region. It is possible that slight differences in apparent electrophoretic mobility of hippocampus and liver mRNAs may be explained by possible differences in polyadenylation or features that influence electrophoretic mobility such as variations between Northern blots.

Northern blots indicated large differences in ACT mRNA levels in liver and hippocampus. Semiquantitative slot blots (Northern blots) with specified amounts of poly(A)+ RNA showed that liver contains approximately 900-fold higher levels of ACT mRNA compared with that in hippocampus (Fig. 4). Northern blots were subjected to autoradiography (15 h exposure to x-ray film) for detection of ACT mRNA.

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Molecular Identity of α-1-ACT in Normal and AD Brains

Fig. 4. Northern blot of ACT mRNA in hippocampus from AD and normal hippocampus as well as liver. Northern blots of total RNA isolated from hippocampus of normal (N) and AD brains (10 μg of RNA each, lanes 1 and 2, respectively) and liver poly(A)+ RNA (1 μg, lane 3) probed with the human ACT cDNA (6), as described under “Experimental Procedures.” Autoradiography of Northern blots (15 h exposure to x-ray film) (lanes 1–3) showed ACT mRNA in hippocampus and a high level of ACT mRNA in liver. Intact ribosomal RNAs were detected by ethidium bromide staining of the RNA samples on denaturing formaldehyde gels (data not shown). kb, kilobases.

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Genomic Blot and Primer Extension—The hippocampus ACT cDNA identified by RT-PCR indicates the existence of the human ACT gene. Further assessment of the ACT gene(s) was obtained by genomic blots. Human genomic DNA was digested with restriction enzymes and probed with the human liver ACT cDNA (6). The genomic blot (Fig. 6) demonstrated the presence of the ACT gene(s) as several DNA bands after digestion of genomic DNA, indicating the presence of at least one or possibly several ACT genes.

To compare transcriptional initiation sites of ACT gene expression in human hippocampus and liver, primer extension analyses were performed. Primer extension of poly(A)+ RNA from hippocampus and liver (lanes 1 and 2, respectively) was conducted with 32P-labeled primer 5′-CTGCCTCAGGGAGCTGGA-3′. The 32P-extended cDNA was analyzed on 8% acrylamide, bis-acrylamide, 7 M urea DNA sequencing gels, with detection of the extended cDNA by autoradiography, as described under “Experimental Procedures.” Arrows indicate the radiolabeled cDNAs of 58 bp obtained by primer extension.

Fig. 5. Slot blot of ACT mRNA in hippocampus and liver. Slot blots of poly(A)+ RNA (with the indicated amounts of RNA) from hippocampus and liver were performed to compare ACT mRNA levels in these two tissues. Hybridization of slot blots with ACT cDNA as probe was performed identically as described for Northern blots of ACT mRNA (Fig. 4). Northern blots were subjected to autoradiography (15 h exposure to x-ray film) for detection of ACT mRNA.

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Deglycosylation of ACT—Multiple glycosylation sites (Asn-Xaa-Thr/Ser) are indicated by the hippocampus ACT cDNA, suggesting that brain ACT exists as a glycoprotein. Therefore,
Fig. 8. Deglycosylation of ACT in hippocampus of Alzheimer’s disease and normal brains as well as in liver. Deglycosylation by N-glycosidase F of ACT in tissue extracts from hippocampus of AD and normal brains as well as human liver ACT was assessed by Western blots with anti-ACT serum. Panel A shows ACT in normal (N) and AD hippocampus (lanes 1 and 3, respectively). ACT in these tissues was also incubated with (+) N-glycosidase F (lanes 2 and 4, respectively). Panel B shows human liver ACT without and with N-glycosidase F treatment (lanes 1 and 2, respectively).

the extent of glycosylation of ACT in hippocampus was examined by deglycosylation with N-glycosidase F, which cleaves the N-glycan linkage of glycoproteins between Asn residues and the carbohydrate chain (19). Western blots (Fig. 8) showed hippocampus ACT of 60–65 kDa in normal and AD brains, which was deglycosylated by N-glycosidase F to a band of approximately 46 kDa. The deglycosylated 46-kDa hippocampus ACT is consistent with the theoretical molecular weight of the ACT polypeptide calculated from its primary sequence, deduced from its cDNA. The liver ACT of 66–75 kDa was slightly larger than the hippocampus ACT. Deglycosylation of liver ACT to a 46-kDa polypeptide indicates that both liver and hippocampus ACT consist of similar molecular weight polypeptide backbones. Differences in apparent molecular weights of hippocampus and liver ACTs and the similar molecular weights of their polypeptide backbones suggest that the two forms of ACT may undergo different types of glycosylation. Overall, these results indicate that the hippocampus and liver ACTs are both glycoproteins.

DISCUSSION

Immunohistochemical detection of the protease inhibitor ACT in amyloid plaques in the hippocampus region of AD brains suggests a role for a protease inhibitor in AD (1, 2). Recent identification of isoforms of neuroendocrine ACTs in bovine adrenal medulla and pituitary (3, 4) and reports of variations in the primary sequences of isolated human liver cDNAs (5–8) lead to the question of the molecular identity of ACT in AD and normal brains. In this study, direct RT-PCR and DNA sequence analyses of hippocampus ACT cDNAs from AD and normal brains show that the hippocampus ACT possesses the reactive site loop that is characteristic of serpins, with Leu as the predicted P1 residue for inhibition of putative brain chymotrypsin-like proteases. The hippocampus ACT cDNAs from control and AD brains were identical in nucleotide and deduced primary sequences and resemble the human liver ACT with greater than 90% homology. Further comparison of ACT in hippocampus and liver showed that ACT gene expression in these two tissues utilizes identical transcription initiation sites. However, significantly lower levels of ACT mRNA are expressed in hippocampus compared with liver. In addition, the hippocampus ACT protein appears to be differentially glycosylated compared with liver ACT. These studies have defined the primary sequence and molecular characteristics of hippocampus ACT expressed in control and Alzheimer’s disease brains.

Before this study, ACT in human hippocampus was thought to resemble human liver ACT based on recognition of the brain ACT with antibodies against human liver ACT (1, 2). However, several reports have indicated variations in nucleotide and deduced primary sequences for full-length and partial human liver ACT cDNAs (5–8). In this study, direct DNA sequence analyses of the hippocampus ACT cDNA indicates that its deduced primary sequence differs by 37 residues compared with the human liver ACT cDNA reported by Chandra et al. (5). However, the hippocampus ACT cDNA is identical to human liver cDNAs characterized by other groups (6–8). These results indicate that the ACT expressed in human hippocampus and human liver ACT (5–8) are nearly identical in nucleotide and deduced primary sequences.

The hippocampus ACT possesses the RSL domain (Fig. 3, boxed region) that participates in the specificity of the serpin to regulate target proteases. The predicted Leu-Ser as P1-P1’ residues are known to inhibit chymotrypsin, suggesting that ACT may inhibit a brain chymotrypsin-like protease. It is noteworthy that cross-class inhibition of cysteine proteases by serpins occurs. For example, the interleukin 1β-converting enzyme (20, 21) and caspase cysteine proteases (22, 23) are inhibited by the CrmA serpin encoded by the cowpox virus (24). In addition, ACT has been demonstrated as a potent inhibitor of a cysteine protease, known as prohormone thiol protease (PTP), that is involved in pro-neuropeptide processing (25). It may, therefore, be logical to predict that ACT in Alzheimer’s disease or normal brains may regulate a serine or cysteine protease.

The characterization of a single hippocampus ACT cDNA is consistent with the demonstration of the human ACT gene(s), as demonstrated by genomic blots. Furthermore, primer extension analyses showed that ACT gene expression in hippocampus and liver utilizes the same transcription initiation site. However, the hippocampus possesses significantly lower levels of ACT mRNA than liver, with differences of approximately 900-fold. These findings suggest differential tissue regulation of ACT gene expression in hippocampus compared with liver. In addition, examination of ACT mRNAs by Northern blots showed that hippocampus and liver ACT mRNAs were close in size, 1.5–1.6 kilobases, and possess nearly identical 3’-UTRs.
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Subsequent to translation of the ACT mRNA, ACT undergoes posttranslational modification as a glycoprotein. Differential glycosylation demonstrated by sensitivity to N-glycosidase F suggests that there may be different types of glycosylation for the ACT in hippocampus compared with liver. Hippocampus and liver ACT proteins appear as 60–65-kDa and 66–75-kDa proteins, respectively, on SDS-polyacrylamide gel electrophoresis. After deglycosylation by N-glycosidase F, the ACT polypeptide backbone is represented by a 45–46-kDa band in both tissues. The apparent molecular mass of deglycosylated ACT is consistent with the theoretical molecular mass of mature ACT of 46,248 daltons, calculated from the ACT cDNA.

The cellular localization of ACT is an important consideration for future studies of proteases that may be regulated by ACT, because protease inhibitor(s) and target protease(s) must be co-localized to allow their interaction in vivo. The NH2-terminal signal sequence of ACT suggests cellular routing of the ACT to the secretory pathway. The predicted transport of ACT within neurosecretory vesicles from neuronal cell bodies along the axon to nerve terminals is supported by the disappearance of ACT from nerve terminals following axotomy (26). Subsequent to secretion, extracellular ACT accumulates with β-peptide in amyloid plaques of AD brains (1, 2). Target proteases regulated by ACT in brain may be colocalized with intracellular or extracellular ACT. Knowledge of the molecular identity of ACT obtained in this study will allow future identification of target proteases that may be regulated by ACT in normal and AD brains.

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