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Purification, cDNA cloning, functional expression, and characterization of a 26-kDa endogenous mammalian carboxypeptidase inhibitor

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ABSTRACT **The recent demonstration of the occurrence in rat brain and other nonpancreatic tissues of carboxypeptidase A (CPA) gene transcripts without associated catalytic activity could be ascribed to the presence of a soluble endogenous protein inhibitor. This tissue carboxypeptidase inhibitor (TCI), detected by the inhibition of added bovine pancreatic CPA, was purified from rat brain. Peptides were obtained by partial proteolysis of purified TCI, a protein of ≈30 kDa, and starting from their sequences, a full-length cDNA encoding a 223-amino acid protein containing three potential phosphorylation sites was cloned from a cDNA library. Its identity with TCI was shown by expression in *Escherichia coli* of a recombinant protein recognized by antibodies raised against native TCI and displaying characteristic CPA-inhibiting activity. TCI appears as a hardly reversible, noncompetitive, and potent inhibitor of CPA1 and CPA2 ($K_i \approx 3$ nM) and mast-cell CPA ($K_i = 16$ nM), which is less potent against carboxypeptidase B (CPB; $K_i = 194$ nM) and inactive on various other proteases. This pattern of selectivity might be attributable to a limited homology of a 11-amino acid sequence with sequences within the activation segments of CPA and CPB known to interact with residues within their active sites. The widespread expression of TCI in a number of tissues (e.g., brain, lung, or digestive tract) and its apparently cytosolic localization point to a rather general functional role, e.g., in the control of cytosolic protein degradation.**

Proteinases participate in a large variety of cell processes, namely, in modeling and communication, but their activity could be detrimental if not tightly controlled. The activity of many proteinases is controlled by the occurrence of endogenous protein inhibitors as is the case for matrix metalloproteinases with which such inhibitors may form hardly reversible complexes (1). For other proteinases, the control mechanism is the necessary cleavage of a segment of a propeptide to release the mature proteinase, endowed with catalytic activity, a scheme that applies, for instance, to the pancreatic carboxypeptidases (2–4).

These two major control mechanisms are not mutually exclusive but, for carboxypeptidases A and B (CPA and CPB, respectively), the only known protein inhibitors—i.e., the inhibitors from *Solanacea* (5, 6) or *Ascaris lumbricoides* (7)—are not of mammalian origin.

We have recently characterized in brain and several other extrapancreatic tissues the presence of CPA1 and CPA2 gene transcripts that were not accompanied, however, by any typical CPA catalytic activity, even using extremely sensitive detection systems (8). For CPA2, this discrepancy was explained by the fact that the transcript corresponds to a splice variant leading to a shorter CPA2 isoform with modified CPA activity. In contrast, for CPA1, a mRNA encoding the full-size pancreatic propeptide was characterized but no CPA activity could be generated after incubation of tissue extracts in presence of trypsin under conditions that should have severed the "activation segment" and released the mature CPA. The discrepancy was explained, in this case, by detection, in extracts from brain and other tissues expressing the CPA1 gene, of an endogenous inhibitory activity directed toward added bovine pancreatic CPA. The latter was rather thermostable but sensitive to the action of Pronase.

In the present work, we have purified this tissue carboxypeptidase inhibitor (TCI) from rat brain, cloned the corresponding cDNA,† and studied its properties and tissue distribution.

MATERIALS AND METHODS

Materials. CPA (from bovine pancreas, 50 units/mg of protein), CPB (from porcine pancreas, 105 units/mg of protein), carboxypeptidase Y (CPY, from baker's yeast, 100 units/mg of protein), leucine aminopeptidase (from porcine pancreas, 140 units/mg of protein), phenylmethylsulfonyl fluoride, *o*-phthaldialdehyde, hippuryl-L-phenylalanine (HF), *N*-ethylmaleimide, and leupeptin were supplied by Sigma; aminopeptidase M (from hog kidney, 20 units/mg of protein) was from Pierce; α-chymotrypsin (from porcine pancreas, 20 units/mg of protein), trypsin (from bovine pancreas, 10,900 units/mg of protein), and elastase (from porcine pancreas, 85 units/mg of protein) were from Boehringer Mannheim; and Ala-AMC and other AMC substrates were from Bachem (AMC is 7-amido-4-methylcoumarin).

Radioactive materials were from Amersham and other materials were from commercial sources.

Enzyme Activity Determinations. CPA activity was determined by using the radioiodinated Bolton–Hunter reagent derivative of the dipeptide Arg-Phe as substrate (8). Briefly, samples (50 μl) were incubated with radioiodinated Bolton–Hunter reagent-coupled Arg-Phe (10^5 dpm, 50 μl), the reaction was stopped by adding 0.2 M HCl (50 μl), and liberated ^{125}I-labeled Bolton–Hunter reagent was recovered by reverse-phase chromatography (Porapak Q, Millipore). Alternatively, the substrate hippuryl-[^{3}H]phenylalanine ([^{3}H]HF, 10^5 dpm/50 μl) was used, and [^{3}H]phenylalanine formed in presence of CPA was also isolated. CPA activity was also determined fluorimetrically, with HF as substrate and *o*-phthaldialdehyde as fluorogenic reagent (9).

CPB (EC 3.4.17.2), carboxypeptidase H (CPH, EC 3.4.17.10), carboxypeptidase M (CPM, EC 3.4.17.12), and CPY (EC 3.4.16.14) activities were measured with hippuryl-[^{3}H]arginine as

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Abbreviations: CPA, CPB, etc., carboxypeptidase A, carboxypeptidase B, etc.; TCI, tissue carboxypeptidase inhibitor; HF, hippuryl-L-phenylalanine; AMC, 7-amido-4-methylcoumarin; RT–PCR, reverse transcription-coupled PCR.

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†The sequence reported in this paper has been deposited in the GenBank data base (accession no. U10260).

substrate and [^{3}H]arginine was isolated on a polystyrene bead column with water as eluent. CPH activity was measured from AtT20 cells culture medium (10). CPM activity was assayed from human lung membranes (11). CPY activity was assayed in 50 mM potassium phosphate (pH 6.0) using the commercial enzyme.

Neprilysin (enkephalinase, EC 3.4.24.11) activity was evaluated as described (12). Peptidyl dipeptide hydrolase (angiotensin-converting enzyme, EC 3.4.15.1) activity was evaluated from human kidney membranes with 0.2 mM *o*-aminobenzoylglycyl-*p*-nitrophenylalanyl proline as substrate (13). Aminopeptidase M (EC 3.4.11.2), leucine aminopeptidase (EC 3.4.11.1), and leukotriene A4 hydrolase (EC 3.3.2.6) aminopeptidase activities were assayed from commercial enzymes with 25 μM Ala-AMC in 50 mM Tris·HCl (pH 7.4). Trypsin (EC 3.4.21.4), α-chymotrypsin (EC 3.4.21.1), and elastase (EC 3.4.21.36) activities were assayed with 2.5 μM Z-Arg-AMC, Ala-Ala-Phe-AMC, and Suc-Ala-Ala-Ala-AMC as substrates, respectively (where Z is benzyloxycarbonyl and Suc is succinyl).

TCI Activity. Tissue samples were preincubated for 1 h in presence of purified bovine CPA and the resulting activity was evaluated by using ^{125}I-labeled Bolton–Hunter reagent-coupled Arg-Phe or [^{3}H]HF as substrate.

Isolation of TCI from Rat Brain. Brains from 10 male adult Wistar rats (Iffa-Credo) were homogenized in 30 ml of 50 mM Tris·HCl (pH 7.5). The supernatant (25 ml) resulting from a centrifugation at 50,000 × *g* for 20 min was precipitated with $(NH_4)_2SO_4$ (10–60% saturation), solubilized, and dialyzed against Tris buffer. The dialysate was submitted to chromatofocusing on a PBE94 column (10 × 1 cm) eluted with PB74 buffer (Pharmacia). Fractions containing CPA inhibitory activity were pooled (100 ml), brought to 1 M $(NH_4)_2SO_4$, and loaded onto a phenyl-Sepharose (Pharmacia) column (10 × 1 cm) equilibrated in the same buffer. Elution was carried out by a linear gradient from 1 M $(NH_4)_2SO_4$ to 50 mM Tris·HCl (pH 7.5). The fraction with TCI activity (5 ml) was then submitted to gel filtration onto a Superdex 75HR 10/30 column (Pharmacia). Active fractions were submitted to SDS/PAGE and proteins were transferred to Immobilon-P membranes (Millipore) in buffer (50 mM sodium borate/50 mM Tris) at 25 V overnight. Membranes were stained for 5 sec with 0.1% amido black in 1% AcOH and rinsed extensively with water. A colored spot, at 30 kDa, enriched proportionally to the inhibitory activity of the tested fractions, was excised and microsequenced.

Peptide Microsequencing. Excised spots were digested overnight with endoproteinase Lys-C at 37°C, in 100 mM Tris·HCl (pH 9.0) containing 10% (vol/vol) CH_3CN. The released peptides were separated on a C_{18} μBondapak HPLC column with a 0–55% CH_3CN gradient in 0.1% trifluoroacetic acid. Peptides were microsequenced by Edman degradation on an Applied Biosystems model A470 sequencer with an on-line 120A phenylthiohydantoin HPLC analyzer.

cDNA Cloning. Probes for library screening were obtained by PCR using degenerate primers derived from the peptide sequence. A single-strand cDNA was synthesized from 2 μg of rat brain poly(A)$^+$ mRNA (14), with avian myeloblastosis virus reverse transcriptase (25 units, for 2 h at 42°C) and a degenerated antisense primer (mixture of 32 different 20-mers, 2 μM), in 20 μl of a mixture containing all four dNTPs (Boehringer Mannheim; each at 2.5 mM), 2.5 mM dithiothreitol, 50 mM Tris·HCl (pH 8.3), 40 mM KCl, 6 mM $MgCl_2$, and RNase inhibitor (40 units). This template was amplified in 0.1 ml of a reaction mixture containing the degenerated sense primer (mixture of 48 different 20-mers, 0.4 μM), 1.5 mM $MgCl_2$, 50 mM KCl, 0.01% gelatin, and 1 unit of *Taq* polymerase, for 35 cycles, the annealing step being at 46°C. The reaction products were resolved by gel electrophoresis (2% NuSieve agarose and 1% agarose), and the band of interest was extracted. The DNA was purified by using a Geneclean II kit, blunt-ended with T4 DNA polymerase, phosphorylated, and subcloned in a *Sma* I-digested pGEM-4Z.

A probe generated by reverse transcription-coupled PCR (RT–PCR) from rat brain poly(A)$^+$ mRNA by using degenerated primers designed from peptide 2 (see Fig. 3) was used to screen a rat hypothalamic cDNA library (1 × 10^6 phages) constructed on λZAP II (Stratagene). This probe (1 × 10^6 dpm/ml) was hybridized in 40% (vol/vol) formamide/4× SSC/1× Denhardt's solution/8 mM Tris·HCl, pH 7.4/yeast tRNA (20 μg/ml)/0.1% SDS/salmon sperm DNA (20 μg/ml)/10% (wt/vol) dextran sulfate. Clones were plaque-purified and Bluescript KS(+) plasmids containing cDNA inserts were recovered using the helper phage R508 and sequenced (15). The sequence of 3′-end region was completed by using a rapid amplification of cDNA ends system (16).

Expression in *Escherichia coli*. An 890-bp PCR fragment containing the entire open reading frame was subcloned in pUC9 plasmid. Transformed bacteria (*E. coli* HB101; Invitrogen) were grown and diluted to an OD_{600} of 0.1. After 18 h, cells were resuspended by sonication in 2 ml of phosphate buffer containing leupeptin (2 μg/ml), 1 mM phenylmethylsulfonyl fluoride, and 5 mM 2-mercaptoethanol and then centrifuged at 10,000 × *g* for 20 min. The recombinant protein was isolated from the supernatant by $(NH_4)_2SO_4$ precipitation and chromatofocusing.

SDS/PAGE and Western Blot Analysis. Proteins after SDS/PAGE in 12% polyacrylamide gels were either stained with Coomassie brilliant blue or blotted onto nitrocellulose sheets. Blots were revealed with IgGs obtained from rabbits immunized against TCI purified from rat brain and a ^{125}I-labeled secondary antibody (17).

Northern Blot Analysis. Total or poly(A)$^+$ mRNAs (14) were subjected to electrophoresis in 1% formaldehyde/agarose gel, blotted onto nitrocellulose, and hybridized at 42°C in 40% formamide/2× Denhardt's solution/50 mM Tris·HCl, pH 7.4/4× SSC/0.1% sodium pyrophosphate/1% SDS/denaturated salmon sperm DNA (0.1 mg/ml)/yeast tRNA (50 μg/ml) with a nick-translated [^{32}P]cDNA probe (3.5 × 10^6 dpm/ml). Blots were washed for two 15-min periods in 2× SSC/0.1% SDS and for two 15-min periods in 0.2× SSC/0.1% SDS at 42°C.

RESULTS

Isolation and Microsequencing of TCI. Preliminary studies showed TCI activity from cerebral homogenates to be recovered in soluble form. Therefore, the high-speed supernatant of a brain extract was used as a source for purification. After $(NH_4)_2SO_4$ precipitation, chromatofocusing showed a single

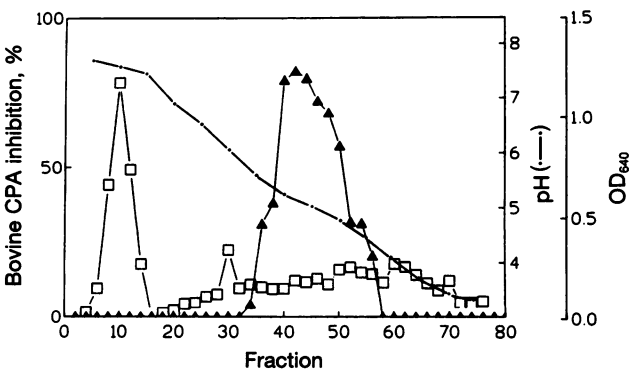

FIG. 1. Chromatofocusing pattern of TCI. TCI activity (triangles) was evaluated by inhibition of a standard bovine CPA activity. The fraction pH is noted with a dotted line. The protein content (squares) was estimated as the OD at 640 nm, after the addition of Coomassie blue.

Table 1. Purification steps of TCI from rat brain

Step	Inhibitory activity (IC_{50}), ng/ml	Purification coefficient	Yield, %
Soluble fraction	70,000	1.0	100
Ammonium sulfate	50,000	1.4	97
Chromatofocusing	2,000	35	81
Phenyl-Sepharose	1,000	70	49
Gel filtration	200	350	16

peak of TCI with an isoelectric point between pH 5.5 and 5.0 (Fig. 1). Polybuffer 74 was eliminated and active fractions were concentrated through phenyl-Sepharose chromatography (Table 1). Finally, gel filtration led to a single inhibitory fraction with an apparent M_r of ≈30,000. Analysis of the fraction by SDS/PAGE revealed a major band at 30 kDa, the intensity of which was correlated to the inhibitory activity (data not shown), with a purity of ≈50%. Western blot analysis indicated a single 30-kDa band (Fig. 2). TCI, isolated by excision, was blotted onto a nylon membrane and digested by endoproteinase Lys-C. Among the 30 peptides generated, three were sequenced, one of them containing 34 amino acids (peptide 2 in Fig. 3).

Cloning of a cDNA Corresponding to TCI. Two partially degenerate oligonucleotides were designed from peptide 2 to perform RT–PCR: the sense primer was a mixture of 48 20-base oligonucleotides, corresponding to the peptide MGQGSAP, and the antisense primer, a mixture of 32 20-mers, corresponding to the peptide FYQRLMS (Fig. 3). By using rat brain poly(A)$^+$ mRNA as template, a fragment of ≈100 bp was generated by RT–PCR, blunt-end ligated in the pGEM-4Z, and sequenced, confirming that it encoded peptide 2. It was then ^{32}P-labeled and used to screen a rat hypothalamic cDNA library. Among 18 cloned phages, one (λ13, 1020 bp) contained an open reading frame of 669 nt. Conceptual translation of this clone revealed a protein of 223 amino acids (M_r = 25,580) that contains the peptide sequences 1–3 obtained by microsequencing (Fig. 3). The 646-bp 3′ end of another clone (λ18, 1200 bp) was homologous to that of λ13, but the upstream sequence diverged from it at position 152. The divergent sequence around position 152 (CCAAAAG/CCACCC) displayed a potential intron–exon structure (18). The intron localization was confirmed by RT–PCR carried out with rat brain mRNA and either a λ13 sense primer (control) or the λ18 corresponding primer. The former amplification led to the expected signal, whereas the latter did not (data not shown). The λ13 insert ended with a CCACTAATA sequence

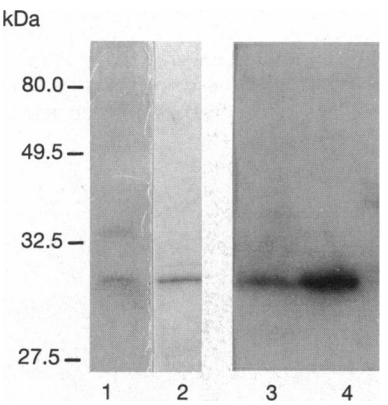

FIG. 2. SDS/PAGE of semipurified and recombinant TCI. Coomassie brilliant blue staining with 1 μg of semi-purified (lane 1) and recombinant TCI (lane 2) and Western blot analysis showing the immunological similarity between native (lane 3) and recombinant TCI (lane 4) are shown. Molecular masses (kDa) of markers are indicated.

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gcccagcccagctgctgacttcaccacctcaccgagacccactccgctggcc   52

agcacgctacactgacaggatcctggctgcacccactgtgatctctttgaac  104

taggatagcccgcgctcactgtttccctgaag  ATG GAA GAA ATC CCA  151
     *                             M   E   E   I   P      5
↓                                                  ♦
CCC ACC CAC TAC GCT GCG TCC AGG GCT GCC TCG GTG GCA     190
 P   T   H   Y   A   A   S   R   A   A   S   V   A      18

GAG AAC TGT ATA AAT TAT CAG CAA GGG ACC CCC AAC AAG     229
 E   N   C   I   N   Y   Q   Q   G   T   P   N   K      31

GTA TTC AAG GTG CAG ACG GTC CAG CAG GCA AGC AAG GAG     268
 V   F   K   V   Q   T   V   Q   Q   A   S   K   E      44

GAT ATC CCA GGA AGA GGC CAC AAG TAC CAT CTG AAG TTT     307
 D   I   P   G   R   G   H   K   Y   H   L   K   F      57
♦
TCT GTG GAA GAA ATT ATC CAA AAA CAA GTT ACA GTG AGC     346
 S   V   E   E   I   I   Q   K   Q   V   T   V   S      70

TGC ACG GCT GAA GTT CTT TAC CCT CGA ATG GGA CAA GGC     385
 C   T   A   E   V   L   Y   P   R   M   G   Q   G      83
                                     ■
TCT GCA CCG GAA GTC AAC TTC ACA TTT GAA GGA GAA ATC     424
 S   A   P   E   V   N   F   T   F   E   G   E   I      96

GGC AAG AAC CCA GAT GAG GAA GAC AAC ACG TTT TAT CAA     463
 G   K   N   P   D   E   E   D   N   T   F   Y   Q     109
             ♦
AGA CTC ATG TCC ATG AAG GAA CCA CTA CAA GCA CAA AAT     502
 R   L   M   S   M   K   E   P   L   Q   A   Q   N     122
                             ■
ATT CCA GAC AAT TTT GGA AAT GTG TCT CCA CAA ATG AAG     541
 I   P   D   N   F   G   N   V   S   P   Q   M   K     135

CCG GTC CAC CAC TTA GCC TGG GTC GCC TGT GGT TAT GTA     580
 P   V   H   H   L   A   W   V   A   C   G   Y   V     148
                     ■
ATG TGG CAG AAC TCG ACT GAA GAC ACA TGG TAT AAA ATG     619
 M   W   Q   N   S   T   E   D   T   W   Y   K   M     161

GCA AAG ATT CAA ACG GTC AAG CAA GTG CAA AGA AAC GAT     658
 A   K   I   Q   T   V   K   Q   V   Q   R   N   D     174

GAC TTC ATT GAG TTA GAC TAC ACC GTC CTA CTC CAC GAC     697
 D   F   I   E   L   D   Y   T   V   L   L   H   D     187

GTT GCA TCG CAG GAG ATT ATC CCC TGG CAA ATG CAA GTT     736
 V   A   S   Q   E   I   I   P   W   Q   M   Q   V     200

CTG TGG CAC CCA CAA TAC GGC ACA AAA GTA AAG CAC AAC     775
 L   W   H   P   Q   Y   G   T   K   V   K   H   N     213

AGC CGC CTC CCA AAG GAA GCA CCA GCA GAG  taaacaagacc   816
 S   R   L   P   K   E   A   P   A   E   *             223

ccaactcggagacagccgtcaacatgccccttaaccatgagtatgactgtcc   868

tcactggcacctagctaacaggccaagtgaatcccacagtgtcgctctgtat   920

gtcttggctacaggaccgagctgcatagattccacaataaagagtagactac   972

atgaaagtgtctttccaatagtaatggtactatggctatccactaataaagt  1024

aacttcagcctcacaaaaaaaaaaaa                            1050
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FIG. 3. Nucleotide and deduced amino acid sequences of the TCI cDNA. The first nucleotide presented in the sequence follows the *Eco*RI cloning site (data not shown). The two terminal codons (TAG at the 5′ and TAA at the 3′ ends) are marked by an asterisk (*). The intron position (nt 152) is indicated with a solid arrow, and the two polyadenylylation signals are underlined. The three microsequenced peptides, in boldface type, are shown in boxes: peptide 1 (dashed-line box), peptide 2 (light-lined box), and peptide 3 (thick-lined box). The three glycosylation sites are indicated with solid squares and the potentially phosphorylated serines are indicated with solid diamonds.

that could have represented the beginning of a polyadenylylation signal sequence. A 3′-end rapid amplification of cDNA ends PCR (16) carried out with rat brain poly(A)$^+$ mRNA as template, and a $(dT)_{17}$ antisense and 5′-CCCAACTCGGAGACAGCCGTCA-3′ sense primers led to a 234-bp fragment. It was subcloned and sequenced, confirming the presence of two polyadenylylation signals separated by 55 bases. The number of nucleotides between the second polyadenyly-

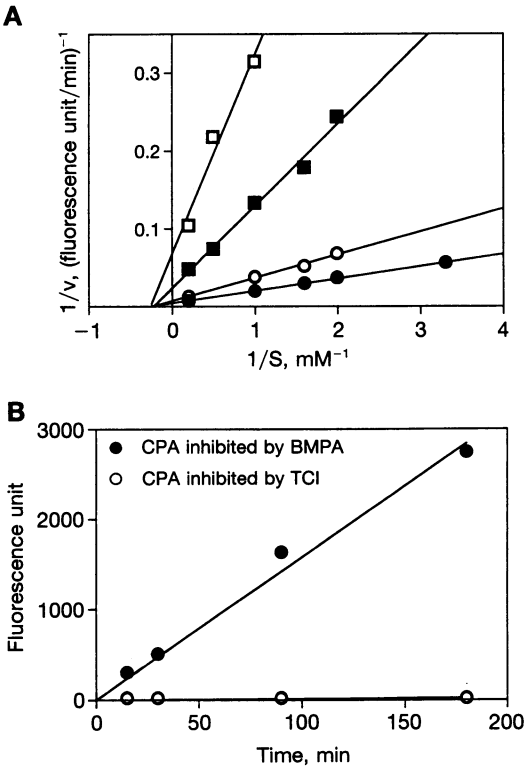

FIG. 4. Inhibition of purified bovine CPA by TCI. (*A*) Lineweaver–Burk plot for inhibition of purified bovine CPA (6 nM) by TCI. The velocity was determined fluorometrically using HF (0.3–5 mM) as substrate. Recombinant TCI concentrations were 0 (●), 3 nM (○), 7.5 nM (■), and 10 nM (□). (*B*) Activity of purified bovine CPA, inhibited by 2 μM 2-benzyl-3-mercaptopropanoic acid (BMPA) (●) and 100 nM TCI (○) with 30 mM HF as substrate.

lation signal and the poly(A) tail was 16 bp, corresponding to the consensus distance between these sites.

Properties of Recombinant TCI. After the transformation of an *E. coli* strain with a 890-bp cDNA encoding TCI, the protein was obtained in nearly pure form by combination of $(NH_4)_2SO_4$ precipitation and chromatofocusing. The recombinant TCI exhibited, like TCI purified from rat brain, an apparent molecular mass of 30 kDa and was recognized on Western blots by antibodies raised against the latter (Fig. 2).

The purified recombinant TCI inhibited bovine CPA with an IC_{50} value of 2 nM (data not shown) and in an apparently noncompetitive manner (Fig. 4*A*). To determine whether TCI exhibited a reversible effect on the enzyme, purified bovine CPA was incubated for 1 h with recombinant TCI (100 nM, i.e., 30-fold the apparent K_i), and an excess of substrate (30 mM, HF) was added. TCI afforded complete inhibition, whereas 2-benzyl-3-mercaptopropanoic acid [2 μM, i.e., 200-fold the K_i

Table 2. Inhibitory activity of recombinant TCI toward various peptidases

Enzyme	IC_{50}, nM
Bovine CPA	2.0 ± 0.3
Rat CPA1	3.2 ± 0.4
Rat CPA2	3.5 ± 0.7
Rat mast cell CPA	16 ± 4
Bovine CPB	194 ± 22

Rat CPA2 (short isoform), mouse CPH, human CPM, porcine aminopeptidase M, porcine leucine aminopeptidase, human neprilysin, human peptidyl dipeptide hydrolase, human leukotriene A4 hydrolase, yeast CPY, bovine trypsin, porcine α-chymotrypsin, and porcine elastase tested under the same experimental procedures were not inhibited (i.e., $IC_{50} > 1$ μM).

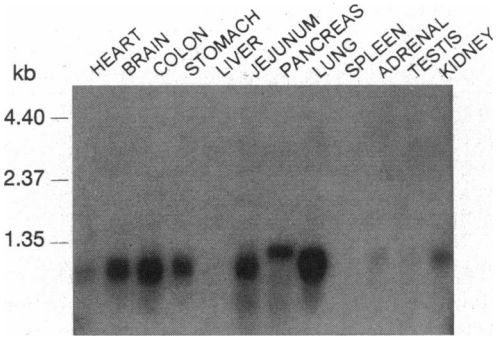

FIG. 5. Northern blot analysis of TCI mRNA in various tissues. Poly(A)$^+$ mRNAs (8 μg per lane) were used. The blot was hybridized with a 102-bp ^{32}P-labeled probe (3.5×10^6 dpm/ml), corresponding to peptide 2 cDNA (Fig. 3), and exposed for 7 days at −80°C with intensifying screens. Molecular sizes (kb) are indicated.

(19)], a reversible inhibitor, did not (Fig. 4*B*). CPA1 and CPA2 from rat pancreas were inhibited with similar potencies, whereas inhibition of other carboxypeptidases was less potent and other metallopeptidases or serine proteases were not significantly inhibited (Table 2). Very similar IC_{50} values against all these peptidases were generated with the inhibitor purified from rat brain (data not shown).

Expression of TCI in Rat Tissues. Northern blot analysis performed with either a 102- or 480-bp cDNA probe (corresponding to fragments of nt 373–475 and nt 277–757, respectively) showed a single major signal corresponding to 1.2 kb in a variety of peripheral tissues (Fig. 5) and brain regions (Fig. 6).

DISCUSSION

We describe herein the amino acid sequence and distribution in rat tissues of TCI, an endogenous metallopeptidase inhibitor that does not appear to belong to any of the known families of proteins endowed with proteinase inhibitory activity (20).

TCI was purified nearly to homogeneity from rat brain, a tissue in which its biological activity was detected (8). SDS/PAGE analysis of fractions obtained during purification pointed out an enrichment in a 30-kDa band, which was excised and used to obtain polyclonal antibodies. The final preparation, displaying a 350-fold enrichment in CPA-inhibitory activity, revealed, on Western blots, a major band of ≈30 kDa representing ≈50% of the total protein. Assuming that it corresponded to TCI, this band was excised and proteolytically digested, and some of the resulting peptides were microsequenced. Semidegenerate 20-mer oligonucleotides derived from a 34-amino acid peptide were selected (21) as primers to generate by RT–PCR a 102-base probe with rat brain poly(A)$^+$ mRNA as template. This probe was then used to screen a

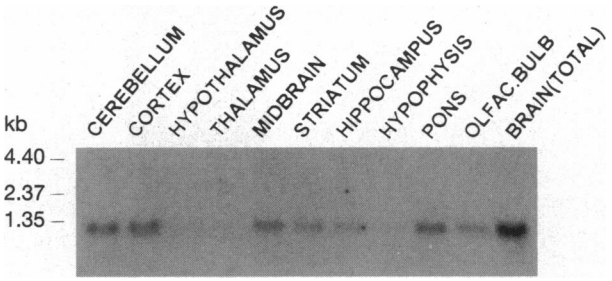

FIG. 6. Northern blot analysis of TCI mRNA in various brain structures. Total RNAs (6 μg per lane) were used. The blot was hybridized with a 480-bp cDNA probe (5×10^6 dpm/ml) and exposed for 14 days at −80°C with ^{32}P intensifying screens. Molecular sizes (kb) are indicated.

cDNA library from rat hypothalamus and a 1020-bp cDNA with an open reading frame of 669 nt was cloned and its poly(A) sequence was completed by RT–PCR. Its potential coding sequence starts at an ATG codon (137) surrounded by an appropriate consensus sequence (22).

Several features indicate that the 223-amino acid sequence encoded by this cDNA corresponds to that of TCI. (*i*) It contains sequences of two additional microsequenced peptides derived from the purified TCI preparation. (*ii*) It encodes a protein of 223 amino acids with a calculated molecular weight of 25,580. The apparent size of either natural or recombinant TCI, by SDS/PAGE analysis, was ≈30 kDa. (*iii*) More importantly, expression in *E. coli* led to a recombinant protein recognized on Western blots by polyclonal antibodies raised against purified TCI and displaying a biological activity closely similar to that of the latter in terms of proteinase inhibitory potential. TCI was characterized by potent inhibition of several carboxypeptidases, except a shorter splice variant of CPA2 with modified sensitivity to inhibitors (8), whereas other metalloproteinases or serine proteinases were not affected.

Hence, TCI appears to be a 223-amino acid protein, with three potential glycosylation sites, two potential Ca^{2+}/calmodulin-dependent protein kinase sites (23) (Ser-16 and -113), and one cGMP-dependent protein kinase phosphorylation site (Ser-58). The absence of any signal peptide and potential transmembrane domain is consistent with the apparent cytosolic localization of TCI in brain and other tissues (8). Its sequence displays no significant homology with those of other classes of endogenous proteinase inhibitors of mammalian origin, e.g., plasminogen activator inhibitor (24) or tissue inhibitors of metalloproteases (25–27). In addition, no significant homology was found with either the 39-amino acid residue CPA inhibitor from potato and other *Solanacea* (5, 6) or the 65-amino acid residue inhibitor from *Ascaris lumbricoides* (7). The potato inhibitor is a globular protein with three disulfide bridges binding tightly to CPA (28) that cleaves the C-terminal glycine residue of CPA (29). In contrast, TCI has no predicted cystine bridge and its C-terminal dicarboxylic residue does not make it an optimal substrate for either CPA or CPB. However, a limited degree of homology (46%) was found between 11-residue sequences of TCI starting from Leu-201 (LWHPQYGTKVK) and the "activation segment" of CPB starting from Phe-37 (FWKPDSATQVK). When attached, the 95-residue activation segment of pro-CPB inhibits the carboxypeptidase activity of the enzyme completely and the critical role of the sequence surrounding Asp-41 was proposed (3, 4). This residue is linked to Arg-127 of CPB by a water-mediated bridge and by a salt bridge to Arg-145 that plays a key role in the fixation of peptide substrates. Although the activation segments of CPA and CPB reach only 32% overall identity, they display a very similar structure in this part of the molecule, which, in both cases, plays the same role in carboxypeptidase activity inhibition within the propeptide. When severed from the proenzyme, the two activation segments of CPA and CPB differ, however, in that the former is still a selective and potent inhibitor (K_i = 2 nM) of CPA whereas the latter is inactive (3, 4).

The hypothesis that interaction of TCI with the carboxypeptidases occurs at the same level as their activation segments remains to be tested—e.g., by crystallographic methods or site-directed mutagenesis. In addition, whereas the activation segment inhibits carboxypeptidase activity in a competitive fashion, consistent with its binding to the active site, the inhibition by TCI is hardly reversible and apparently noncompetitive.

TCI gene transcripts were identified in a variety of tissues including brain in which their distribution among regions was somewhat heterogeneous. In all these tissues, a soluble CPA-inhibiting activity and a short CPA2 isoform lacking the activation segment were detected (8). TCI, however, being ineffective on this isoform, it seems unlikely that its function is to inhibit its activity in the cell cytoplasm. Nevertheless, the role of TCI might be related to prevention of degradation of cytoplasmic proteins by other proteases that remain to be identified. In addition, post-translational modifications of TCI (e.g., phosphorylation on its three consensus phosphorylation sites) might modify its biological activity. Additional studies are obviously required to circumscribe the functional role of TCI.

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