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Cloning of a cDNA encoding an ectoenzyme that degrades thyrotropin-releasing hormone

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ABSTRACT **Thyrotropin-releasing hormone (TRH) is an important extracellular signal substance that acts as a hypothalamic-releasing factor, which stimulates the release of adenohypophyseal hormones and functions as a neurotransmitter/neuromodulator in the central and peripheral nervous system. The inactivation of TRH after its release is catalyzed by an ectoenzyme localized preferentially on neuronal cells in the brain and on lactotrophic pituitary cells. This enzyme exhibits a very high degree of substrate specificity as well as other unusual properties. The activity of the adenohypophyseal enzyme is stringently controlled by estradiol and thyroid hormones, indicating that this enzyme itself may serve regulatory functions. Fragments of the enzyme isolated from rat or pig brain were generated by enzymatic digestion or cyanogen bromide cleavage, purified by reverse-phase HPLC, and sequenced. PCR amplification and screening of cDNA libraries from rat brain and pituitary led to the identification and isolation of a cDNA that encodes a protein of 1025 amino acids. The analysis of the deduced amino acid sequence was consistent with the identification of the enzyme as a glycosylated, membrane-anchored Zn metallopeptidase. Furthermore, Northern blot analysis demonstrated that the mRNA levels paralleled the tissue distribution of the enzyme and that in pituitary tissue the transcript levels rapidly increased when the animals were treated with triiodothyronine. Finally, transient transfection of COS-7 cells with this cDNA led to the expression of an active ectopeptidase that displayed the characteristics of the TRH-degrading ectoenzyme.**

Thyrotropin-releasing hormone (TRH) was originally isolated as a hypothalamic hypophysiotropic-releasing factor that stimulates the release of adenohypophyseal hormones (for review, see refs. 1 and 2). Subsequently, TRH was found to be widely distributed throughout the central and peripheral nervous systems as well as in extraneuronal tissues (for review, see refs. 3 and 4). TRH receptors have been identified not only in the pituitary but also in various brain regions (for review, see refs. 5 and 6). Neurochemical and electrophysiological investigations as well as biochemical and pharmacological studies suggest that in extrahypothalamic brain areas TRH most likely acts as a neurotransmitter and/or neuromodulator (for review, see refs. 7–9). Such functions certainly imply the existence of a highly efficient inactivation system to clear the target site for the rapid transmission of the peptidergic signals. There is considerable evidence that the inactivation of neuronally released TRH is catalyzed by a peptidase (for review, see ref. 10) that exhibits, among other unusual characteristics, a very high degree of substrate specificity (11–13), as does the TRH-degrading serum enzyme (14). The particulate peptidase is localized in synaptosomal (11) and adenohypophyseal (15) membrane fractions. In brain-derived cells in culture, it is found only on the surface of neuronal cells but not on glial cells (16–18). In the pituitary the enzyme is localized preferentially on lactotrophs (17), and recent studies have demonstrated that the activity of the adenohypophyseal enzyme is regulated by estradiol (19) and is stringently controlled by thyroid hormones (20–22).

After solubilization by limited proteolysis under very mild conditions, we recently succeeded in purifying the enzyme from rat and pig brain (about 200,000-fold) to electrophoretic homogeneity. In this report we describe the isolation and sequence of a cDNA encoding the TRH-degrading ectoenzyme.‡ Furthermore, we present information on the transcriptional regulation of the adenohypophyseal enzyme by thyroid hormones as well as on the functional expression of this peptidase in transfected mammalian cells.

MATERIALS AND METHODS

Enzyme Fragmentation and Peptide Sequencing. After isolation from rat or pig brain as described elsewhere (23), the TRH-degrading enzyme (50 μg ≈430 pmol) was dialyzed against water, adjusted to 70% formic acid, and exposed in the dark to cyanogen bromide (8 mg in a final volume of 300 μl) for 8 h. After adding water (2 ml), the solvent was evaporated *in vacuo*. Alternatively, 50 μg of the enzyme preparation in 100 μl of buffer (25 mM Tris·HCl, pH 8.5/1 mM EDTA/0.2 M urea/20 mM methylamine) was boiled for 5 min and then incubated at 25°C for 18 h with either 2 μg of endoproteinase Lys-C or 2 μg of trypsin (sequence grade enzymes; Boehringer Mannheim). Aliquots of the reaction mixtures were supplemented with trifluoroacetic acid (0.2%) and subjected to reverse-phase HPLC on C_4 or C_8 Vydac columns. Elution was carried out by increasing (1% per min) the concentration of acetonitrile (5–70% in 0.1% trifluoroacetic acid) in the eluant. Isolated fragments were analyzed by gas-phase sequencing.

Library Construction and Screening. Initially, a rat brain λZAPII cDNA library, generously provided by S. Morley and D. Richter (University of Hamburg, F.R.G.), and a commercially available rat brain λgt10 cDNA library (Clontech) were analyzed by high-stringency screening using random-primed ^{32}P-labeled cDNA fragments (24). In addition, a cDNA library was constructed from the pituitaries of Sprague–Dawley rats that had been treated with triiodothyronine (T_3; 30 μg/100 g of body weight, injected i.p.) 6 h before sacrifice. Poly(A)$^+$ RNA (10 μg) was isolated directly from the pituitary homogenate with the aid of magnetic $(dT)_{25}$ polystyrene beads (Dynal) and used for DNA synthesis employing a ZAP cDNA synthesis kit (Stratagene). After size-fractionation using Sephacryl S-400 spin columns, the cDNA was ligated into Uni-ZAP XR vector arms and packaged according to the manufacturer's instructions (Stratagene), yielding ≈1.5×10^6 independent recombinants. Positive clones were plaque-

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Abbreviations: TRH, thyrotropin-releasing hormone; T_3, triiodothyronine.

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‡The sequence reported in this paper has been deposited in the GenBank data base (accession no. X80535).

Peptide 1	Pig†	YGNAARNDL
	Rat†	YGNAARNDLX NXL
Peptide 2	Pig†	SYDGVAAASF SRAVETVEAN V
	Rat‡	XVETVEAN VRXK
Peptide 3	Pig†	MLYQDELFQ
	Rat†	RLYQDELFQW
	Pig*	LYQDELFQW LGKAL
Peptide 4	Pig*	EKQRFLTDVL HEV
Peptide 5	Pig*	GHSVFQRGLQ DYLTIHKYGN AARNDLWNTL SXA
Peptide 6	Rat*	NSKLISGVTE FLNTEGELKE LKN

FIG. 1. Sequence of peptide fragments. The TRH-degrading ectoenzyme isolated from rat or pig brain was either subjected to cyanogen bromide cleavage (*) or digested with endoproteinase Lys-C (†) or trypsin (‡). The peptide fragments were isolated by reverse-phase HPLC and sequenced.

purified, and the inserts were subcloned into pBluescript KSII+ (Stratagene) and analyzed by the dideoxy chain-termination method employing T7 polymerase (Pharmacia).

PCR Amplification. Five micrograms of poly(A)$^+$ RNA isolated from the pituitaries of T_3-treated rats as described above was reverse transcribed by Moloney murine leukemia virus reverse transcriptase (GIBCO/BRL) (25). Based on the sequence information of the partial cDNA clones from rat brain, three pairs of primers were chosen to amplify the entire coding region. Generally, 100 ng of cDNA was subjected to 50 cycles of amplification employing *Pfu* DNA polymerase (Stratagene). The products were isolated, subcloned, and sequenced as described above.

Northern Blot Analysis. Total RNA was isolated from lung, heart, brain, kidney, and liver from adult Sprague–Dawley rats by homogenization in guanidinium thiocyanate followed by centrifugation through cesium chloride (26). Poly(A)$^+$ RNA was selected by chromatography on oligo(dT) cellulose (Stratagene) or isolated directly from the pituitaries of control or T_3-treated animals as described above. Samples of 5 μg of poly(A)$^+$ RNA were size-fractionated by electrophoresis in a denaturing formaldehyde/agarose gel, transferred to a nylon membrane (Schleicher & Schuell), and crosslinked by UV irradiation. cDNA fragments derived from the coding sequence were randomly labeled with ^{32}P to high specific activity (10^9 cpm/μg) and used as a probe. The same membrane was subsequently stripped and reprobed with cDNA fragments of glyceraldehyde-3-phosphate dehydrogenase and β-actin (Clontech).

Southern Blot Analysis. Rat genomic DNA was digested with an excess of restriction endonucleases and size-fractionated by electrophoresis in 0.8% agarose gels. The fragments were denatured, transferred to a nylon membrane, and hybridized with different cDNA fragments.

Expression in COS-7 Cells. COS-7 cells (5×10^4 cells per dish, 60 mm i.d.) were transfected by the calcium phosphate coprecipitation method (27) with 10 μg of the eukaryotic expression vector pEQ-176P2 (28) containing or not containing a 3.6-kb cDNA fragment that made up the entire coding region of the TRH-degrading ectoenzyme. After transfection and 72 h in culture, the cells were washed three times with 5 ml of DMEM and incubated in 2.5 ml of DMEM containing 5 μCi (1 Ci = 37 GBq) of [*pGlu*-^{3}H]TRH (2 μM) and the inhibitors of the cytosolic TRH-degrading enzymes pGlu-CHN_2 (2 μM) (29) and Cbz-Gly-Pro-CHN_2 (4 μM) (30), which do not affect the activity of the TRH-degrading ectoenzyme (pGlu = pyroglutamic acid and Cbz = benzyloxy carbonyl). After incubation at 37°C for given periods of time, aliquots (100 μl) of the culture medium were removed to determine the degradation of TRH by the radiochemical test described in ref. 17. Thereafter, the cells were washed three times with 5 ml of DMEM, collected, and homogenized by sonication in 2.5 ml of the same incubation mixture that was used before. Alternatively, the transfected cells were washed three times with 5 ml of DMEM and incubated for 15 min at 4°C with 1 μg of trypsin (Boehringer Mannheim) in 2 ml of DMEM containing 100 mg of bovine serum albumin. After adding egg white trypsin inhibitor (4 μg in 10 μl of DMEM), the medium was aspirated, supplemented with 500 μl of a mixture containing pGlu-CHN_2 (10 μM), Cbz-Gly-Pro-CHN_2 (20 μM), and 5 μCi of [^{3}H]TRH, and incubated at 37°C to determine the activity of the TRH-degrading ectoenzyme.

RESULTS

Peptide Sequences of the TRH-Degrading Ectoenzyme. Peptide fragments of the isolated TRH-degrading ectoenzyme from rat and pig brain were generated either by cyanogen bromide cleavage or by enzymatic digestion with endoproteinase Lys-C or trypsin. Surprisingly, treatment with trypsin was rather ineffective and resulted mainly in fragments of high molecular weight that could not be resolved by reverse-

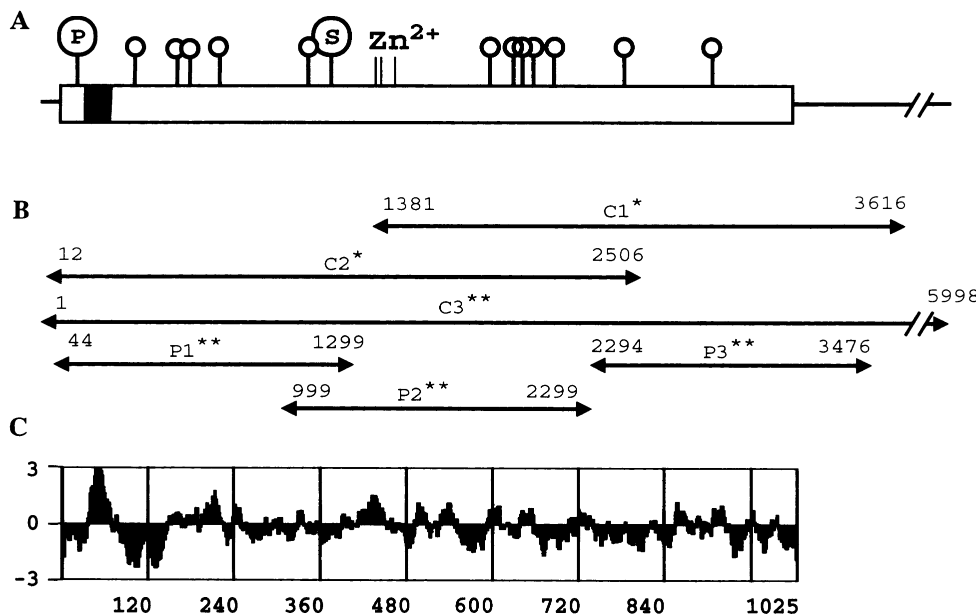

FIG. 2. Illustration of the isolated cDNAs and schematic structure of the deduced protein. (*A*) The straight line represents the untranslated regions, and the box represents the open reading frame. The presumed transmembrane-spanning sequence is represented by the black box. The potential protein kinase C phosphorylation site (P), the putative tyrosine sulfation site (S), the 12 potential N-glycosylation sites, and the Zn-binding motif are indicated. (*B*) Summary of the isolated clones (C) and PCR amplification products (P) from brain (*) and pituitary (**) cDNA. The numbers at the beginning and the end of each fragment indicate the position relative to the longest clone isolated. (*C*) Hydrophobicity profile of the amino acid sequence encoded by the cDNA of the TRH-degrading ectoenzyme as calculated with an average window of 16 amino acids. The ordinate and abscissa represent the hydrophobicity and the amino acid sequence, respectively.

phase HPLC. Thus, only a few small peptides could be isolated. In contrast, digestion with endoproteinase Lys-C under the same conditions was considerably more effective. Best results were obtained when the enzyme was subjected to cyanogen bromide cleavage. Several fragments could be isolated and sequenced (Fig. 1).

cDNA Cloning. Based on the amino acid sequences of peptides 1 and 2, a PCR experiment with degenerate primers has been performed, which led to the amplification of a cDNA fragment (31). This fragment lacked a continuous reading frame but coded for the additional amino acids that had not been used for the primer construction (31). Using this fragment as a probe, screening of 1.5×10^6 recombinants from the brain λZAPII cDNA library led to the identification of two independent clones that accounted for 3.6 kb of cDNA.

PCR experiments were performed with pituitary cDNA to verify the composite sequence resulting from the brain cDNA clones. Three primer pairs were chosen to amplify the entire open reading frame. Products of about 1.2 kb each were obtained as single bands. In addition, a cDNA clone of 6 kb could be isolated from 1.0×10^6 recombinants from the pituitary cDNA library (Fig. 2*B*). Sequencing of both the brain and the pituitary cDNA revealed that the overlapping nucleotide sequences were identical.

cDNA Sequence Analysis. Fig. 3 presents the nucleotide sequence and the deduced amino acid sequence of the TRH-degrading ectoenzyme. Although no stop codon was found at the 5′ end, it can be assumed that the translation initiation site is located at nucleotide 73, since this is the first ATG in the isolated cDNA clones and it also matches the criteria for a consensus initiator methionine (GCCACCATGGGA) as analyzed by Kozak (32). The first in-frame stop codon (TAA) is located at nucleotides 3148–3150. A peculiar stretch of 27 uninterrupted AT repeats is found further downstream (nucleotides 3918–3972). A consensus sequence for polyadenylation (AATAAA) is present at nucleotides 5471–5476. The resulting open reading frame of 3075 nucleotides codes for a protein containing a polypeptide core of 1025 amino acids. The calculated mass of 117,302 Da is in good agreement with the estimated molecular mass of the deglycosylated TRH-degrading ectoenzyme as determined by SDS/PAGE.

Hydropathy analysis using the Kyte and Doolittle algorithm (33) predicts a transmembrane-spanning domain near the amino terminus (Fig. 2*C*). This stretch of 22 hydrophobic amino acids is preceded by a basic stop transfer sequence,

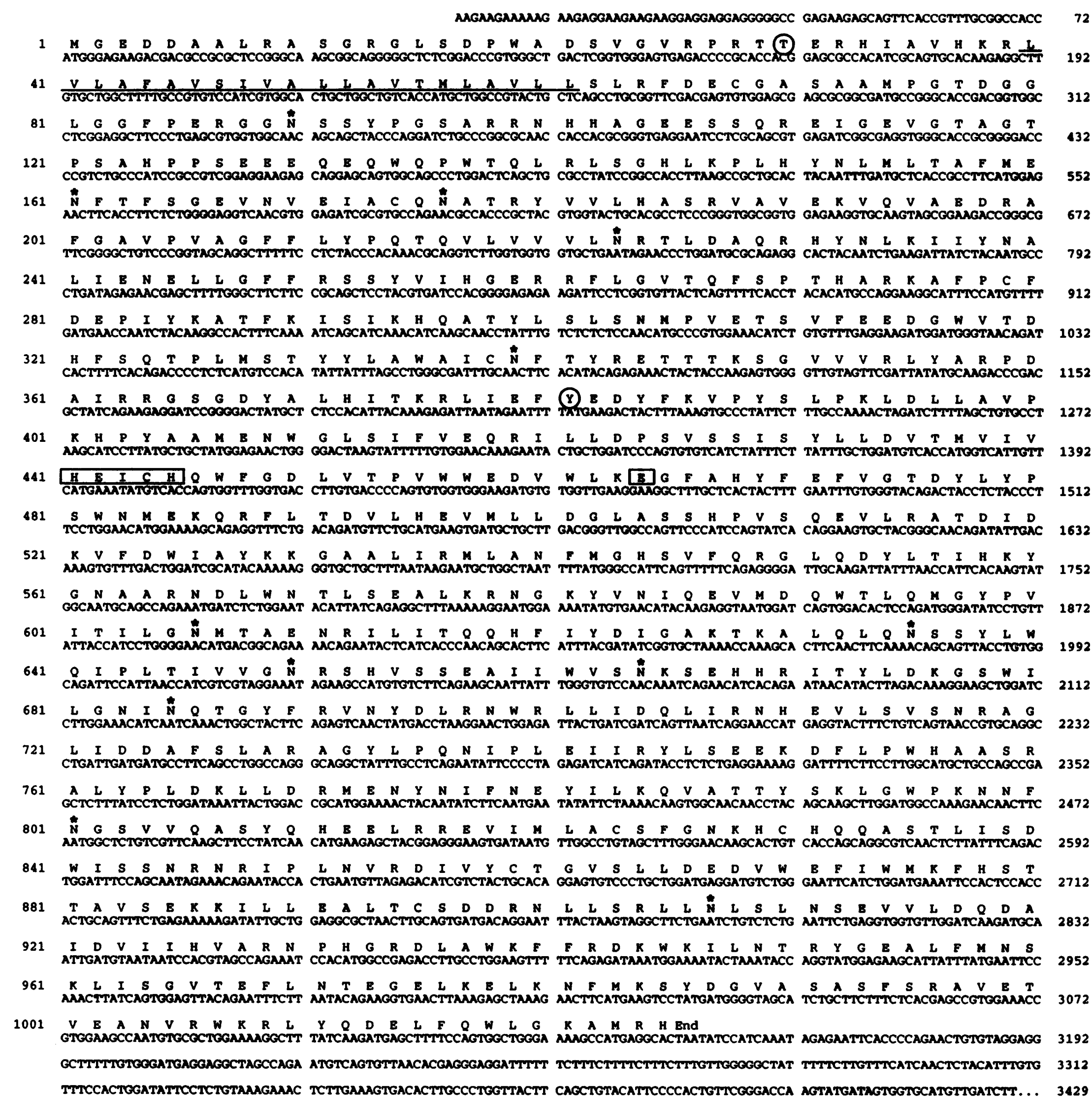

FIG. 3. Nucleotide sequence of the composite cDNA and the deduced primary structure of the TRH-degrading ectoenzyme. The putative transmembrane-spanning sequence is underlined, the potential glycosylation sites are marked by an asterisk, and the putative tyrosine sulfation site (amino acid residue 381) and the potential protein kinase C phosphorylation site (amino acid residue 30) are encircled. The HEXXH consensus sequence and the conserved glutamic acid (amino acid residue 464) are boxed.

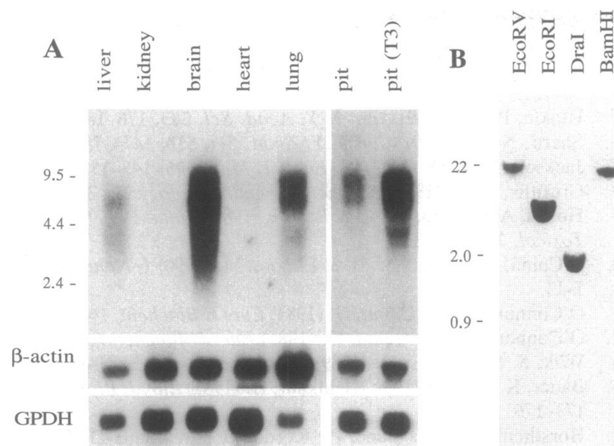

FIG. 4. Northern blot analysis of TRH-degrading ectoenzyme (*A*) and Southern analysis of the corresponding gene (*B*). (*A*) Poly(A)$^+$ RNA (5 μg per lane) from rat liver, kidney, brain, heart, and lung as well as from pituitaries (pit) of euthyroid or T_3-treated animals was probed with ^{32}P-labeled cDNA fragments from the open reading frame of the TRH-degrading ectoenzyme. The β-actin and glyceraldehyde-3-phosphate dehydrogenase (GPDH) signals obtained from the same blots are presented to provide information as to the quality of the analyzed mRNA preparations. The molecular sizes (kb) indicated at left were determined by using an RNA ladder (GIBCO/BRL). (*B*) Rat genomic DNA (7 μg per lane) was digested by various restriction endonucleases and probed with a 254-bp fragment (nucleotides 916–1170) of the TRH-degrading ectoenzyme.

indicating that the TRH-degrading ectoenzyme is a type II integral membrane protein anchored by an uncleaved signal sequence (34). The amino-terminal and presumably intracellular part of the enzyme also contains a threonine residue, which might be a potential site for phosphorylation by protein kinase C (35). Within the large extracellular domain, a consensus sequence for tyrosine sulfation (amino acid 381) (36) and 12 putative N-glycosylation sites (37) are found. Most importantly, this domain also contains the consensus sequence of the zinc-dependent metallopeptidase family—namely, the HEXXH motif (38) at positions 441–445 with a second glutamic acid separated by 18 amino acids (39).

Tissue Distribution. Northern blotting revealed that rat brain and pituitaries contain several transcripts of the TRH-degrading ectoenzyme ranging in size from approximately 6 to 9.5 kb (Fig. 4). Weaker signals were also detected with mRNA from lung and liver but not from heart and kidney. Already 6 h after injecting euthyroid rats with T_3, a considerable increase in the transcript levels of the TRH-degrading ectoenzyme became evident in the pituitary (Fig. 4*A*) but not in the other tissues tested (brain and liver).

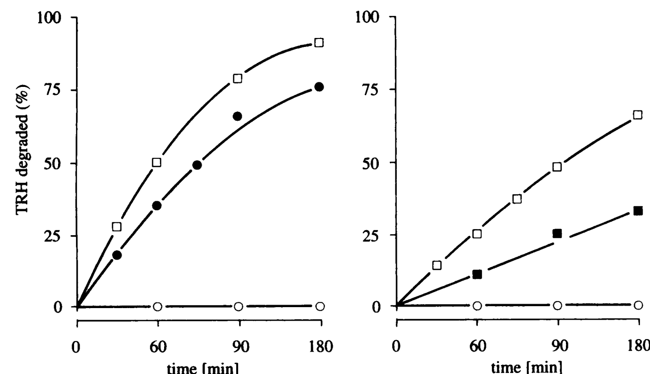

FIG. 5. Degradation of TRH by transfected COS-7 cells. Radiolabeled TRH was added to the culture medium of COS-7 cells transfected either with the expression vector (pEQ176P2) containing the cDNA of the TRH-degrading ectoenzyme (●, □, ■) or with the unmodified plasmid (○). The degradation of TRH was determined under the conditions described in *Materials and Methods* by directly following the degradation of radiolabeled TRH added to the cell culture medium (●), by determining the activity of the enzyme in the homogenates of the transfected cells (□), or by assaying the enzymatic activity that was proteolytically released into the culture medium after incubation with trypsin (■).

Southern Analysis. Several fragments from different regions of the cDNA were used for Southern analysis with genomic DNA from Sprague–Dawley rats. The simple pattern of single bands (Fig. 4*B*) supports the notion that the TRH-degrading ectoenzyme is encoded by a single gene.

Expression of the TRH-Degrading Ectoenzyme. The degradation of TRH by transfected COS cells was determined under the enzyme-specific conditions described in *Materials and Methods*. Rapid degradation of TRH was only observed in the medium of cells that were transfected with the expression vector containing the cDNA of the TRH-degrading ectoenzyme but not in the culture medium of control cells (Fig. 5). Under all conditions, $<0.05\%$ of the total radioactivity was found in the cell lysates, indicating that neither TRH nor the radiolabeled split product pGlu was taken up by these cells. Moreover, comparable enzymatic activities were found when the respective cell homogenates were tested. These findings strongly suggest that the enzyme is localized on the surface of the transfected cells. Furthermore, about 50% of the total enzymatic activity could be released from the transfected cells by treatment with trypsin under the mild conditions that were used to solubilize the TRH-degrading ectoenzyme from rat and pig brain. Like the isolated brain peptidase, the recombinant enzyme was inhibited by EDTA in a time-dependent manner but not by general inhibitors such as diisopropyl fluorophosphate and 2-iodoacetamide.

DISCUSSION

We describe here the isolation and the sequence of a cDNA that encodes the TRH-degrading ectoenzyme. This conclusion is based on the finding that the data obtained at the molecular level are fully compatible with the data obtained previously by biochemical studies. The deduced amino acid sequence contains all peptide sequences that were obtained by enzymatic and chemical fragmentation of the purified enzyme. Moreover, the cDNA predicts a protein that contains a transmembrane-spanning domain, potential glycosylation sites, and the HEXXH consensus sequence of metallopeptidases with an additional glutamic acid residue 18 amino acids apart, which constitute the active site of metallopeptidases (38, 39). These features are in complete agreement with the biochemical identification of the TRH-degrading ectoenzyme as a glycosylated, membrane-anchored Zn metallopeptidase (40). Furthermore, transfection of eukaryotic cells with an expression vector containing the isolated cDNA resulted in the synthesis of an enzymatically active protein that seems to be inserted properly in the plasma membrane as an ectoenzyme.

The biochemical significance of the putative sulfation and phosphorylation sites remains to be determined. While protein sulfation might be important for intracellular sorting and trafficking, the potential phosphorylation of the cytoplasmic threonine residue by protein kinase C could be of regulatory importance. Previous studies (41) on the inactivation of the TRH-degrading ectoenzyme on Y-79 retinoblastoma cells by phorbol esters have suggested that the inactivation of the enzyme might correlate with its phosphorylation by protein kinase C. Similarly, the phorbol ester-induced down-regulation of neutral endopeptidase 24.11 on the surface of neutrophils has also been ascribed to the phosphorylation of this peptidase by protein kinase C (42).

In complete agreement with the activities of the TRH-degrading ectoenzyme (16, 43, 44) is the analysis of the

Northern blots, which demonstrated that the mRNA levels follow a very unusual tissue distribution. Highest transcript levels are found in the brain, whereas the kidneys (generally the richest source of peptidasic activities such as aminopeptidase N, aminopeptidase A, and neutral endopeptidase 24.11) do not contain detectable amounts of this mRNA or significant enzymatic activities. Although multiple mRNA forms are detected by Northern blotting, the Southern analysis indicates that the TRH-degrading ectoenzyme is present as a single-copy gene. This finding also supports the previous notion (11) that the serum TRH-degrading enzyme and the TRH-degrading ectoenzyme, which exhibit identical chemical properties, are derived from the same gene. As a working hypothesis, we assume that the TRH-degrading serum enzyme, which seems to be secreted by the liver, may be formed by alternative splicing.

As expected from the previous studies on the tissue-specific regulation of the adenohypophyseal TRH-degrading ectoenzyme by thyroid hormones (20–22), the basal mRNA levels of the pituitary enzyme were very low but rapidly increased when the animals were treated with T_3.

A comparison of the complete amino acid sequence of the rat TRH-degrading ectoenzyme with those in the translated GenBank (December 1993) revealed significant homology with the sequences of aminopeptidase N (45–47) (34% to the rat enzyme) and mouse aminopeptidase A (32%) (48). Both enzymes have been extensively characterized and identified as membrane-anchored Zn metallopeptidases (for review, see refs. 49 and 50). While the intracellular parts and the transmembrane-spanning regions are completely different, there are sequences in the extracellular domain that display a high degree of homology, and two stretches each with 7 amino acids are completely conserved, indicating that these enzymes share common ancestry. These findings were somewhat surprising since the amino peptidases and the TRH-degrading ectoenzyme are strikingly different with respect to their tissue distribution, the cellular localization, and substrate specificity.

The homology to other proteins is rather limited. Even when the HEXXH consensus sequences of all metallopeptidases are aligned, only the expected homology within this family is found. However, among these peptidases the TRH-degrading ectoenzyme is unparalleled in that it contains a cysteine residue within the zinc-binding motif. The functional significance of this cysteine residue with respect to the substrate specificity of the TRH-degrading ectoenzyme remains to be investigated (e.g., by site-directed mutagenesis). It is hoped that the isolation of the cDNA encoding the TRH-degrading ectoenzyme will open new possibilities to study the structure, the biological function, and the regulation of this interesting enzyme at the molecular level.

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