We have isolated by immunological screening of a λZAPII cDNA library constructed from rat brain mRNAs a cDNA clone encoding endopeptidase 3.4.24.16. The longest open reading frame encodes a 704-amino acid protein with a theoretical molecular mass of 80,202 daltons and bears the consensus sequence of the zinc metalloprotease family. The sequence exhibits a 60.2% homology with those of another zinc metalloproteinase, endopeptidase 3.4.24.15. Northern blot analysis reveals two mRNA species of about 3 and 5 kilobases in rat brain, ileum, kidney, and testis. We have transiently transfected COS-7 cells with pcDNA3 containing the cloned cDNA and established the overexpression of a 70–75-kDa immunoreactive protein. This protein hydrolyzes QFS, a quenched fluorimetric substrate of endopeptidase 3.4.24.16, and cleaves neurotensin at a single peptide bond, leading to the formation of peptides with a molecular mass of 2747 (11–13). QFS and neurotensin hydrolysis are potently inhibited by the selective endopeptidase 3.4.24.16 dipeptide blocker Pro-Ile and by dithiothreitol, while the enzymatic activity remains unaffected by phosphoramidon and captopril, the specific inhibitors of endopeptidase 3.4.24.11 and angiotensin-converting enzyme, respectively. Altogether, these physicochemical, biochemical, and immunological properties unambiguously identify endopeptidase 3.4.24.16 as the protein encoded by the isolated cDNA clone.

Endopeptidase 3.4.24.16 is a metalloendopeptidase ubiquitously distributed in the central nervous system and in peripheral organs of mammals. This enzyme was first detected (2) and later purified (3) on the basis of its ability to cleave the Pro10–Tyr11 bond of the tridecapeptide neurotensin, leading to the formation of the biologically inactive catabolites, neuroten- sin (1–10) and neurotensin (11–13). Studies on neurotensin catabolism in vitro by membrane fractions or cell lines of central or peripheral origin indicated that endopeptidase 3.4.24.16 was the only peptidase that ubiquitously contributed to the inactivation of this neuropeptide (4). Several lines of evidence later suggested that endopeptidase 3.4.24.16 indeed participated to neurotensin inactivation in vivo in the gastrointestinal tract (5). Thus, by means of a vascularly perfused model of dog ileum, we showed that the dipeptide Pro-Ile, a fully selective blocker of endopeptidase 3.4.24.16 (6), inhibited the formation of one of the major catabolites, i.e. neurotensin (1–10) (5), leading to a drastic protection of neurotensin from degradation.

In the central nervous system, we recently showed that mixed inhibitors of endopeptidases 3.4.24.16 and 3.4.24.15 potently enhanced the neurotensin-induced analgesia in the hot plate-tested mice (7). Altogether, this indicates that endopeptidase 3.4.24.16 contributes to the catabolism of neurotensin in vivo in the periphery but also likely in the central nervous system.

The characterization of the biochemical and pharmacological properties of endopeptidase 3.4.24.16 indicated that the enzyme behaved as a 70–75-kDa monomer that was inhibited by metal chelators and dithiothreitol (3). Several studies suggested that endopeptidase 3.4.24.16 resembles another metalloproteinase, endopeptidase 3.4.24.15. Particularly, studies on the specificity of endopeptidase 3.4.24.15 showed that the enzyme cleaved several neuropeptides at peptidyl bonds that were reminiscent of those targeted by endopeptidase 3.4.24.16 (8, 9). However, several aspects that included the nature of the cleavage site on neurotensin, the sensitivity to dipeptide inhibitors and dithiothreitol, as well as immunological data clearly distinguished the two peptidases (10). The present paper reports on the molecular cloning and expression of rat brain endopeptidase 3.4.24.16 and establishes that the two peptidases are related but clearly distinct molecular entities.

EXPERIMENTAL PROCEDURES

Materials

Restriction and modifying enzymes and synthetic oligonucleotides were from Eurogentec (Seraing, Belgium). The DNA sequencing kit was from Applied Biosystems. [α-32P]dCTP (3000 Ci/mmol) was purchased from ICN Biomedicals. Nylon membranes were from Amersham Life Science (Buckinghamshire, United Kingdom). Horseradish peroxidase-conjugated antibody and molecular weight markers were obtained from Promega. Mc-Pro-Leu-Gly-Pro-O-Lys-dinitrophenyl (QFS) was from Novabiochem (Meudon, France). Pro-Ile, dithiothreitol, o-phenan-throline, and 4-Chloro-1-naphtol were purchased from Sigma. Phosphoramidon (N-[o-oleoylcarboxamido(2S)-2-phenylethyl]-L-tyrosine) was from Boehringer (Mannheim, Germany). Captopril was from the SQUIBB Institute. Phosphodieryl 20 was synthesized and kindly given by Dr. V. Dive (CEN Saclay, France).

Screening of a Rat Brain cDNA Library and Isolation of the Full-length cDNA

A rat brain cDNA library constructed in the λZAPII vector (Stratagene) was screened with a polyclonal antibody raised against the rat brain endopeptidase 3.4.24.16 (1). Approximately 6 × 106 recombinant phages were plated and incubated at 42 °C for 3.5 h; then each plate was overlaid with a nylon filter previously saturated in 10 mM isopropyl-β-thiogalactopyranoside and incubated at 37 °C for 3 h. The filters were blocked overnight at 4 °C in TBST buffer (150 mM NaCl, 0.05% Tween 20 in 10 mM Tris- HCl, pH 8) containing 5% of fat milk, then incubated for 8 h at 4 °C with a 1/1000 dilution of the primary antibody in TBST containing 1% of fat milk. Filters were washed three times (5 min each) in TBST and incubated for 1 h at room temperature in 1% fat milk TBST containing a 1/2500 dilution of a goat anti-rabbit antibody conjugated with horseradish peroxidase. The antibody-antigen complexes were revealed with the chromogenic substrate 4-chloro-1-naph-
The cDNAs of 14 isolated positive clones were subcloned into pBluescript by in vivo excision according to the manufacturer’s procedures (Stratagene). A clone, λ7a with an insert of 1806 bp, was sequenced and showed an open reading frame of 1613 bp, lacking the 5′-region coding for the N-terminal domain of the protein. Using two synthetic oligonucleotide primers, a polymerase chain reaction fragment of 1396 bp was derived from the λ7a clone, labeled with 32P by random-priming (Amplitigene), and used as a probe to screen 6 × 105 clones of the above λZAPII cDNA library. Hybridization was carried out overnight at 65 °C in 6 × SSC, 0.1% SDS, 5 × Denhardt’s solution, and 0.2 mg/ml heat-denatured herring sperm DNA. The filters were washed in 3 × SSC, 0.1% SDS at room temperature and autoradiographed. A clone, λ7aB1, containing an insert of 2158 bp, was isolated. This clone encompassed 1516 bp of clone λ7a (Fig. 1) but lacks the complete 3′-region as illustrated by the absence of a stop codon. Therefore the full-length cDNA was reconstituted with these two overlapping cDNAs by ligating a 380-bp NcoI–EcoRI fragment of λ7a with a 2210-bp KpnI–NcoI fragment of λ7aB1. The resulting insert, λ7aB1, was subcloned in pBluescript previously digested with KpnI–EcoRI. This construction allowed us to confirm the whole sequence of the cDNA and to verify that the ligation of the two fragments occurred without introduction of errors in the coding phase.

cDNA Sequencing

The automated sequencing was performed by means of the dideoxy chain termination method (11) on both strands by walking along the cDNA using synthetic dinucleotides according to the strategy described in Fig. 1.

Northern Blot Analysis

Poly(A)+ mRNAs were prepared from rat tissues by purification on oligo(dT) columns. 5 µg of poly(A)+ mRNAs were electrophoresed on a 1% formaldehyde/agarose gel, transferred onto a nylon membrane, and hybridized with the polymerase chain reaction probe derived from the λ7a clone described above, in 50% formamide, 5× SSC, 0.1% SDS, 10% dextran sulfate, 1× Denhardt’s solution, and 0.2 mg/ml heat-denatured herring sperm DNA for 15 h at 42 °C. The filter was washed twice at room temperature in 1× SSC, 0.1% SDS and two times at 50 °C in 0.1× SSC, 0.1% SDS. Autoradiography was performed at −70 °C for 3 days.

Transient Expression of Endopeptidase 3.4.24.16 in COS-7 Cells

The λ7aB1 fragment was excised from pBluescript with KpnI–EcoRI and subcloned into the KpnI–EcoRI site of the eukaryotic expression vector pcDNA3 (Invitrogen). Semi-confluent COS-7 cells, grown in 100-mm cell culture dishes, were transfected with 1 µg of pcDNA-7aB1 by the DEAE-dextran precipitation method (12). Negative control was performed in the same conditions with 1 µg of pcDNA vector (mock-transfected). Approximately 48 h after transfection, the cells were collected, washed with 25 mM Tris-HCl, pH 7.5 and buffer containing 250 mM sucrose and 1 mM EDTA, centrifuged for 10 min at 5000 rpm, and homogenized in 5 mM Tris-HCl, pH 7.5. Protein concentrations were determined by the Bradford method with γ-globulin as standard (13).

Endopeptidase 3.4.24.16 Assays

Fluorimetric Analysis of QFS Hydrolysis—QFS (50 µM) was incubated for various times at 37 °C with COS-7 cells protein homogenate (final concentration of 0.1 mg/ml of protein) in a final volume of 100 µl of 50 mM Tris-HCl, pH 7.5, in the absence or in the presence of different concentrations of inhibitors. Incubations were stopped with 2.5 ml of sodium formate, pH 3.7, and fluorimetrically monitored at λ345 nm and λ405 nm as described previously (14).

HPLC Analysis of Neurotensin and QFS Hydrolysis—Neurotensin (2 nmol) and QFS (5 nmol) were incubated for various times at 37 °C with COS-7 cells protein homogenate (final concentration of 0.1 mg/ml of protein) in a final volume of 100 µl of 50 mM Tris-HCl, pH 7.5, in the absence or in the presence of inhibitors. Reactions were stopped, centrifuged for 5 min at 10,000 × g, and the supernatants were HPLC analyzed with the triethylammoniumfluorourea acid/acetone trichloromethyltrifluoroacetic acid/dactinomycin chromatographic system previously described (4).
between the sizes of these two mRNA species reflects a variable length of their non-coding 3' -region, consequently, to two distinct polyadenylation sites. An alternative hypothesis could be that the higher molecular weight mRNA represents an intermediate immature form of the mRNA. Finally, the possibility that the mRNAs encode two distinct proteins could be evoked. However, such a hypothesis is not sustained by our previous data indicating that in whole rat brain homogenate, a tissue that would be expected to contain all the various putative molecular forms of the peptidase, we consistently detected a single immunolabeled protein migrating with the apparent molecular weight of the recombinant peptidase (1).

**Cloning of Rat Brain Endopeptidase 3.4.24.16**

**FIG. 2.** Nucleotide and deduced amino acid sequences of rat brain endopeptidase 3.4.24.16 cDNA. Nucleotides and amino acid residues are numbered on the right column. Three possible initiation sites of the translation are indicated by the three circled methionine residues presented in bold. The stretches of charged amino acids are underlined. The consensus sequence of zinc metallopeptidases is boxed. An asterisk indicates the stop codon (TAA) of the open reading frame.

**FIG. 3.** Hydropathic profile of endopeptidase 3.4.24.16 protein sequence. Hydropathy analysis of the amino acid-deduced sequence was obtained by the method of Kyte and Doolittle (44) with a window size of 10 residues.

**FIG. 4.** Northern blot analysis of endopeptidase 3.4.24.16 mRNA. Poly(A)- RNA (5 µg) from various rat tissues isolated on a 1% formaldehyde/agarose gel, blotted on a nylon membrane, and hybridized with the 32P-labeled polymerase chain reaction fragment derived from a 17a clone as described under "Experimental Procedures." RNA molecular weight markers are indicated in kilobases (kb) on the left.
Cloning of Rat Brain Endopeptidase 3.4.24.16

**DISCUSSION**

The immunological approach used in the present study has led us to isolate a cDNA clone that unambiguously encodes endopeptidase 3.4.24.16. First, the protein overexpressed in transfected COS-7 cells is recognized by the IgG-purified fraction of a specific polyclonal antibody developed toward rat brain endopeptidase 3.4.24.16 (1). Second, transfecant cells hydrolyze two peptides (QFS and neurotensin) at peptide bonds identical with those targeted by purified endopeptidase 3.4.24.16 (3, 16). Third, the catalytic activity of the protein produced by transfecant cells is fully inhibited by Pro-Ile, a dipeptide that selectively blocks endopeptidase 3.4.24.16 (6), and by diithiothreitol (19) in agreement with the pharmacological spectrum previously established for rat endopeptidase 3.4.24.16 (14). Furthermore, the activity remains insensitive to the specific inhibitors of angiotensin-converting enzyme, endopeptidase 3.4.24.11, and endopeptidase 3.4.24.15.

It is interesting to note that transfected cells deave neurotensin at a single peptide bond, giving rise to neurotensin (1–10) and neurotensin (11–13). It was recently suggested that endopeptidase 3.4.24.16 of porcine origin targeted the same peptide bond but also triggered a minor production of neurotensin (1–8) and neurotensin (9–13) (20). The present study clearly indicates that the production of neurotensin (1–8) and neurotensin (9–13) by the purified enzyme from porcine

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**Fig. 5. Western blot analysis of the protein expressed by pcDNA,-7aB1 transfected COS-7 cells.** A, homogenate proteins (10 μg) of pcDNA3 (lane 1) or pcDNA,-7aB1 (lane 2) transfected COS-7 cells were electrophoresed on a 15% SDS-polyacrylamide gel and then blotted onto a nylon membrane. The recombinant protein was labeled with the IgG-purified fraction of an antiserum raised against endopeptidase 3.4.24.16 as described under "Experimental Procedures." Molecular weight standards are indicated in kDa on the right. B, proteins (10 μg) from transfected cell homogenate (total) and subcellular fractions (mb, membrane-associated; sb, soluble; 100,000 × g supernatant) were incubated for 10 h at 37°C in absence (−) or in the presence (+) of 0.15 units of endoglycosidase F as described under "Experimental Procedures." Samples were submitted to SDS-PAGE and Western blot analysis in the conditions described above.

**Fig. 6. Hydrolysis of QFS by pcDNA,-7aB1 transfected COS-7 cells and the effect of Pro-Ile and diithiothreitol.** QFS (50 μM) was incubated for the indicated times at 37°C with 10 μg of protein homogenates from pcDNA3 (C) and pcDNA,-7aB1 (●) transfected COS-7 cells, and then hydrolysis was fluorimetrically monitored as described under "Experimental Procedures." A, Mcc-Pro-Leu release was quantified by comparing the fluorescence with that obtained with known amounts of the synthetic peptide. QFS hydrolysis by pcDNA,-7aB1 transfected COS-7 cells was performed as described under "Experimental Procedures." HPLC analyses indicated that one of the cleavage product eluted with retention time of 3-carboxy-7-methoxycoumarin-Pro-Leu and then hydrolysis was fluorimetrically monitored as described under "Experimental Procedures." Data are expressed as the percent of control fluorescence recovered in absence of competing agent.

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**Table 1.** Summary of the activity of endopeptidase 3.4.24.16.

| Substrate | Activity (μmol/min/mg) |
|-----------|------------------------|
| QFS       | 1.5                    |
| neurotensin (1–10) | 0.5                   |
| neurotensin (11–13) | 0.3                   |
| angiotensin (1–10) | 0.2                   |

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**Graph A** shows the hydrolysis of QFS by pcDNA3-7aB1 transfected COS-7 cells. **Graph B** shows the effect of Pro-Ile and diithiothreitol on the hydrolysis of QFS by pcDNA3-7aB1 transfected COS-7 cells.

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**Image 1** depicts a Western blot analysis of the protein expressed by pcDNA3-7aB1 transfected COS-7 cells. The recombinant protein was labeled with the IgG-purified fraction of an antiserum raised against endopeptidase 3.4.24.16. The recombinant protein was detected in transfected COS-7 cells and not in mock-transfected COS-7 cells.

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**Image 2** illustrates the hydrolysis of QFS by pcDNA3-7aB1 transfected COS-7 cells. The hydrolysis of QFS was monitored fluorimetrically as described under "Experimental Procedures." Data are expressed as the percent of control fluorescence recovered in absence of competing agent.

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**Image 3** shows the production of a specific polyclonal antibody developed toward rat brain endopeptidase 3.4.24.16. The antibody was used to detect the recombinant protein in transfected COS-7 cells.

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**Image 4** represents the production of neurotensin by pcDNA3-7aB1 transfected COS-7 cells. The production of neurotensin was monitored fluorimetrically as described under "Experimental Procedures." Data are expressed as the percent of control fluorescence recovered in absence of competing agent.
We previously established that endopeptidase 3.4.24.16 was predominantly recovered in majority in a soluble form in the brain (23). This also appears to be the case in transfected COS-7 cells as illustrated in Fig. 5B, which indicates a major soluble form of the protein, in agreement with the recovered activity in the two subcellular fractions (not shown). Previous immunological data clearly indicate that a minor fraction of endopeptidase 3.4.24.16 could exist in a genuine membrane-associated form in the brain (24). This hypothesis was reinforced by light and electron microscopic analysis of the localization of endopeptidase 3.4.24.16 in rat mesencephalon (25). Thus, it was shown that endopeptidase 3.4.24.16-like immunoreactivity could be characteristically associated with restricted zones of the plasma membrane of a subpopulation of neurons in the rat substantia nigra and ventral tegmental area (25). Biochemical analysis of the type of association of endopeptidase 3.4.24.16 with the membrane of kidney microvilli indicated that the enzyme was not attached to the membrane by a glycosyl-phosphatidylinositol anchor (21) but partitioned in the detergent phase after Triton X-114 phase separation (21), a physicochemical behavior that appears to be common to various intrinsic membrane proteins (26).

Sequence analysis of endopeptidase 3.4.24.16 does not reveal the clearcut structural requirements generally fulfilled by intrinsic membrane-bound proteins. First, it is not possible to clearly delineate a N-terminal signal peptide that could serve as a membrane anchor, as has been shown for endopeptidase 3.4.24.11 (27). In agreement with this observation, it is noticeable that membrane-associated and soluble forms of endopeptidase 3.4.24.16 comigrate after SDS-PAGE and Western blot analysis experiments (Fig. 5B). Second, although there exist three putative glycosylation sites, deglycosylation experiments performed with the whole homogenate of transfected cells as well as with the membrane-associated and soluble enzymes did not affect the apparent molecular weight of the peptidase (Fig. 5B), in agreement with our previous biochemical data showing that endopeptidase 3.4.24.16 did not bind to various sugar-linked resins (3). However, it is interesting to note that several clusters of hydrophobic residues can be deduced from the hydrophilic profile of the protein that could be responsible for some protein-protein interactions. Furthermore, one can underline the presence of a stretch of charged residues at amino acids 331–335 and 341–348. This could be of importance with respect to a previous work showing that carboxypeptidase E displayed a similar domain rich in charged amino acids (28) that was shown to be responsible for the attachment of the “membrane-bound” carboxypeptidase E counterpart to the plasma membrane (29). Mutagenesis analysis experiments should allow us to examine whether the above possibilities could account for the anchoring of the “membrane-bound” form of endopeptidase 3.4.24.16.

Purified endopeptidase 3.4.24.16 is sensitive to metal chelators such as EDTA and o-phenanthroline (3). We showed that the activity of the apoenzyme could be restored upon incubation with various divalent cations, the most efficient recovery being obtained with zinc (19). The sequence of endopeptidase 3.4.24.16 reveals the presence of an HEGF sequence that confirms that the enzyme belongs to the zinc metalloprotease family (15, 30).

The current knowledge of the biochemical and physicochemical features of endopeptidases 3.4.24.16 and 3.4.24.15 and their specificity toward various neuropeptides underlined that the two enzymatic activities share some similar properties (8, 9). On the other hand, the two peptidases can be distinguished by their distinct cleavage sites for neurotensin (3, 8), their sensitivity to dipeptide inhibitors (16, 23), and by the lack of

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2 B. Vincent, J. P. Vincent, and F. Chedir, submitted for publication.
Cloning of Rat Brain Endopeptidase 3.4.24.16

The isolation of the cDNA clone of endopeptidase 3.4.24.16 from rabbit liver was established (34) and shows 90.3% identity with that of endopeptidase 3.4.24.16. Sequence homology strongly suggests that microsomal metalloendopeptidase corresponds to the rabbit counterpart of rat endopeptidase 3.4.24.16. However, very limited information exists on the specificity of this enzyme toward natural neuropeptides since Kawabata and Davie (35) only reported on the ability of microsomal metalloendopeptidase to cleave a synthetic peptide that mimics the amino acid sequence encompassing the processing site of vitamin K-dependent proteins. Further studies are clearly needed to document the specificity of microsomal metalloendopeptidase with respect to the known properties of endopeptidase 3.4.24.16. The sequence of endopeptidase 3.4.24.16 exhibits 24.2 and 25.6% homology with those of a rat liver mitochondrial intermediate peptide (36, 37) and a dipetidyl carboxypeptidase from Escherichia coli (38), respectively. Finally, the enzyme did not align with the sequences of endopeptidase 3.4.24.11 (39, 40) and angiotensin-converting enzyme (41–43).

The isolation of the cDNA clone of endopeptidase 3.4.24.16 should allow us to express a high amount of the recombinant protein. The design of highly potent inhibitors of endopeptidase 3.4.24.16 and their use to affinity purify the enzyme should allow us to obtain high quantities of pure enzyme. This tool should be of importance to examine the detailed structural features of the enzyme and to envision crystallographic experiments. The cDNA should also prove useful to delineate the putative biological signals that could modulate the level of expression of the peptidase as it seems to occur during the differentiation processes of primary cultured neurons (24).

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