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Differential Expression of Two Cathepsin Es during Metamorphosis-Associated Remodeling of the Larval to Adult Type Epithelium in *Xenopus* Stomach

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Cathepsin E (CE) was purified from the foregut of *Xenopus laevis* tadpoles as a mature dimeric form. The purified enzyme was a typical CE among aspartic proteinases with respect to pH dependence of proteolytic activity, susceptibility to pepstatin, and having *N*-linked high-mannose type oligosaccharide chains. We isolated two cDNAs for the CE (CE1 and CE2) from adult stomach. The amino acid sequence of the N-terminal region of the purified CE coincided with the corresponding sequence predicted from CE1. Northern blot analysis and *in situ* hybridization were performed. The CE1 mRNA was highly expressed in surface mucous cells and gland cells constituting the larval epithelium of the foregut of pro-metamorphic tadpoles. As metamorphosis began and progressed, CE1 mRNA drastically decreased in amount, and subsequently both CE1 and CE2 mRNAs gradually increased. The increase in CE2 mRNA was detected shortly after the increase in CE1 mRNA. The decrease in CE1 expression correlated with degeneration of the larval type epithelium, while the increases in both CE1 and CE2 expression correlated with formation of the adult type epithelium. Thus, cathepsin E gene expression was differentially regulated during metamorphosis-associated remodeling of the larval to adult type epithelium in stomach.

Key words: cathepsin E, metamorphosis, stomach, *Xenopus laevis*.

Cathepsin E (CE) is an aspartic proteinases like pepsin, renin and cathepsin D (CD) (*1*, *2*). Unlike pepsin and renin, however, CD and CE are non-secretory, intracellular enzymes. However, they are different from each other in intracellular localization and tissue distribution. CD is a typical lysosomal enzyme (*3*, *4*), while CE is localized in various subcellular components such as endosome-like vacuoles, the *trans*-Golgi area and lumen of ER of microglia, rough ER of gastric cells, and plasma membranes of red blood cells (*4–6*). Among all aspartic proteinases previously investigated, CE has the unique structural characteristic that it forms a homo-dimer through intermolecular disulfide bonding, and has an *N*-linked high-mannose type oligosaccharide chain or a complex type oligosaccharide chain. No O-linked oligosaccharide chain has been detected in the CE molecule yet (*5*, *7*, *8*). It is well known that CE is localized in various cells, tissues or organs such as gastric mucosa, thymus, spleen and blood cells of mammals. Like pepsin, a high amount of CE has been found in stomach (*2*, *9*, *10*). Recently, some interesting findings were reported, *i.e.* that CE plays a role in the presentation of type II histocompatibility antigen (*11*, *12*), and in the degradation or processing of biologically active peptides or precursors such as tachykinin and neurotensin/neuromedin N precursor peptide (*13*, *14*). In addition, CE is closely related to degeneration of neurons and reactivation of glial cells during the processes of brain aging and ischemia (*15*, *16*).

Regarding anurans, tadpoles are herbivorous, while frogs are carnivorous. It is well known that the carnivorousness of frogs is closely related to the occurrence of digestive enzymes such as pepsin produced in their stomaches. On the other hand, the digestive enzyme(s) of herbivorous tadpole remain unclear. Pepsin is not present in tadpoles. Instead of it, there have been some reports that the foregut of *Xenopus* tadpoles has acidic contents, and cathepsin D-like activity was detected in the contents (*17*, *18*). Recently, another aspartic proteinase, CE, was identified in the foregut of bullfrog *Rana catesbeiana* tadpoles. CE was also detected in stomach of adult frogs (*19*, *20*), purified from the stomach, and well characterized (*21*). It remains to be determined whether aspartic protease(s) such as cathepsin D–like protease or CE function as a digestive enzyme in the foregut of herbivorous tadpoles.

In addition to the difference in the proteolytic enzymes occurring in tadpole foregut and frog stomach, it is well known that transition from tadpole to frog is closely related to the metamorphosis-associated change from the larval to adult type of digestive organ. The foregut of *Xenopus* tadpoles is known to consist of a larval esophagus, an immature gland-like organ called the manicotto gland, and a region comprising from the posterior end of the manicotto gland to bile duct opening (post-manicotto

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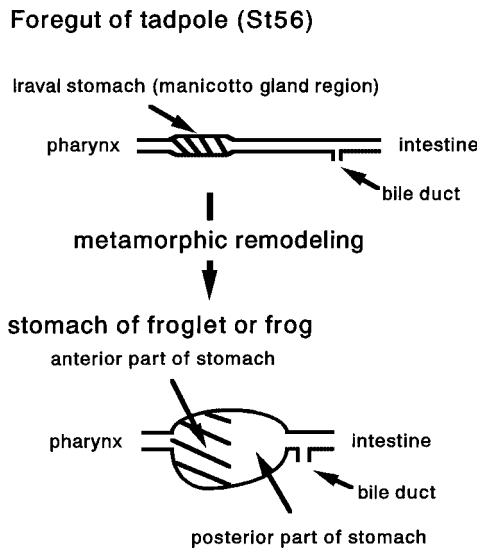

Fig. 1. **Schematic drawing of foregut of tadpoles, and stomach of froglets and adults.** In the present study, the larval foregut is defined as the area from a part posterior to the pharynx to the bile duct opening. The area changes into the esophagus, stomach and duodenum, respectively, after metamorphosis. The larval stomach of pre-metamorphic tadpoles has a gland-like structure called the manicotto gland, while the region just posterior to the stomach to the small intestine has no glandular structure. In this region, a single longitudinal fold, the typhlosole, is found. In frog stomach, the anterior part of the stomach is the gastric gland, and the posterior part has no such glandular structure.

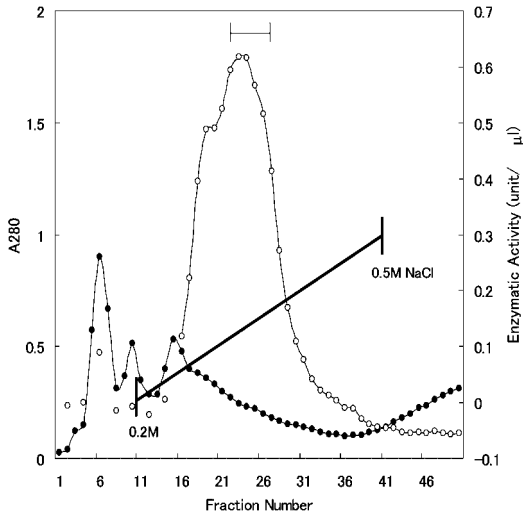

Fig. 2. **Q-Sepharose column chromatography for the purification of cathepsin E.** Twenty ml crude extract of the foregut of *Xenopus* tadpoles was applied to a column (1 × 13 cm) of Q-Sepharose equilibrated with 20 mM Tris-HCl (pH 7.5). After the column had been washed with about 50 ml of the same buffer, elution was performed with a medium consisting of a linear gradient of 0.1 to 0.6 M NaCl buffered with 20 mM Tris-HCl (pH 7.5). Flow rate, 30 ml/h; fraction volume, 3 ml; solid circles, absorbance at 280 nm; open circles, proteolytic activity. The fractions indicated by the bar were pooled and subjected to the next step of purification.

region). The manicotto gland region is the so-called "larval stomach" (See Fig. 1). Histology shows that the larval stomach is composed of epithelium, connective tissue and a thin layer of muscle. The epithelium consists of surface mucous cells and gland cells. As metamorphosis begins and progresses, this larval type epithelium disappears, and is replaced by proliferating and differentiating cells finally constituting the adult type epithelium. At the end of metamorphosis, gastric glands are constructed from the adult type epithelium in the stomach of froglets (*22*). During such metamorphic remodeling, pepsinogens start to be expressed exclusively in oxynticopeptic cells in the gland (*19*, *23*, *24*).

In the present study, we purified and characterized CE in the foregut of *Xenopus laevis* tadpoles, cloned cDNA for CE from adult stomach, and observed the temporal and spatial expression pattern of the CE mRNA during metamorphosis-associated remodeling.

MATERIALS AND METHODS

Animals—Adults and tadpoles of South African clawed frog *X. laevis* were obtained from a commercial supplier. Developmental stages (St) were essentially determined according to the criteria proposed by Nieuwkoop and Faber (*25*). In addition to the Nieuwkoop-Faber's criteria, stages 59 to 61 were strictly determined according to additional histological criteria, as follows: (i) St59, adult epithelial primordia proposed by Ishizuya-Oka *et al*. (*26*) are not still found; (ii) St60, the primordia are first recognized as small spherical cell masses or islets located adjacently to a thin layer of muscle at the basal side of the gut; and (iii) St61, the primordia begin to extensively proliferate, to grow from the basal side toward the luminal side of the gut, and to differentiate into adult type epithelial cells (*24*).

Standard Assay for Cathepsin E Activity, and Determination of the Protein Amount—Using bovine hemoglobin as a substrate, the proteolytic activity of cathepsin E (CE) was measured according to the method previously described (*27*). The amount of protein was estimated using bovine serum albumin as a standard according to the bicinchoninic acid (BCA) method (Pierce). Throughout the purification, the amount of protein was monitored by measuring the absorbance at 280 nm.

Purification of Cathepsin E—Step 1, Preparation of crude extract: Foreguts, *i.e*. the area from the esophagus to the opening of the bile duct, as shown in Fig. 1, were collected from St56–58 tadpoles, washed with 20 mM Tris-HCl buffer (pH 7.5), and the homogenized in the same buffer. The homogenate was centrifuged at 20,000 ×*g* for 30 min. The supernatant, i.e. the crude extract, was used as the starting material for purification of the CE. This procedure was performed at 4°C.

Step 2, Q-Sepharose column chromatography: The crude extract was applied on a column (1 × 13 cm) of Q-Sepharose equilibrated with 20 mM Tris-HCl buffer (pH 7.5). After washing the column with the same buffer, elution was performed with a 150 ml solution consisting of a linear gradient of 0.1 to 0.6 M NaCl buffered with 20 mM Tris-HCl (pH 7.5). This procedure was also performed at 4°C. As shown in Fig. 2, one major and one minor peak of CE activity were discernible. The former contained a mature or active form of CE, and the latter a pro-form of CE.

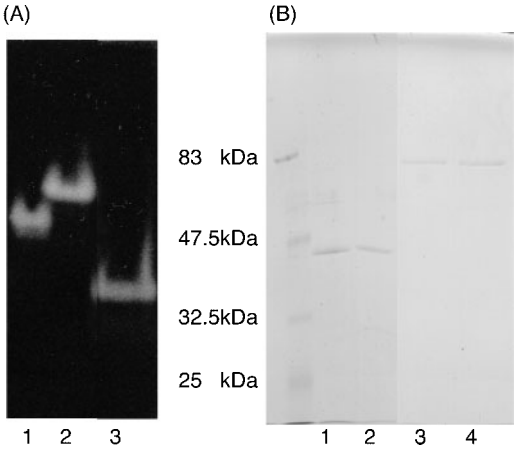

Fig. 3. **Polyacrylamide gel electrophoretic patterns of cathepsin E.** A: Zymograms. Lanes 1, 2 and 3 are zymograms of purified pepsinogen C (PgC, 1 μg), pepsinogen A (PgA, 4 μg), and cathepsin E (CE, 0.5 μg), respectively. Purification of *Xenopus* pepsinogens and zymogram was carried out according to the method previously described (*27*). B: SDS-PAGE patterns. SDS-PAGE was carried out under reducing conditions (lanes 1 and 2) or non-reducing conditions (lanes 3 and 4). Lanes 2 and 4, purified CE; lanes 1 and 3, CE treated with acid (0.5 μg). The acid treatment was performed by the addition of a 1/10 volume of 0.1 N HCl (final pH, 2.4) and incubation at 20°C for 20 min. After electrophoresis, the gel was stained with Coomassie Brilliant Blue R 250.

Step 3, Mono-Q column chromatography: The fractions obtained at step 2 were applied to a column (5 × 50 mm) of Mono-Q equilibrated with 0.15 M NaCl-20 mM Tris-HCl (pH 7.5), and then eluted with a linear gradient of 0.1 to 0.5 M NaCl in a total volume of 40 ml. This step was performed at room temperature. The mature form of CE was purified as a single band on SDS-PAGE and employed for further analyses. Fractions of the pro-form of CE contained some contamination. Therefore, the following procedures were performed: The pro-form separated from the crude extract by SDS-PAGE was electrically blotted onto a PVDF membrane. The membrane comprising only the band of the pro-form were directly subjected to amino acid sequence determination.

Characterization of the Proteolytic Activity of CE—The pH dependence of the proteolytic activity of the purified CE was examined using 2.5% bovine hemoglobin solutions, the pH of which had previously been adjusted to 1.0 to 5.0 with 1.0 N HCl. The inhibitory effect of pepstatin on the activity was examined by the method previously described (*21*).

Digestion of CE with Endoglycosidase H—CE was treated with 0.5 milliunits of endoglycosidase H (Genzyme, MA, USA) in 50 mM phosphate buffer (pH 6.5) containing 0.1% SDS, incubated for 15h at 37°C, and then analyzed by SDS-PAGE (*28*). Protein bands on the gel were visualized with a silver staining kit (Wako, Osaka).

Determination of the Amino Acid Sequence of the NH2-Terminal Region of CE—The partial amino acid sequence of the N-terminal region of CE was determined by Edman degradation using an automatic gas-phase protein sequencer, 476A Protein Sequencer (ABI).

Cloning of cDNAs for Cathepsin E—cDNA was cloned from an RNA fraction of adult stomach using a Marathon cDNA Amplification kit (CLONTEC, Palo Alto, USA), and then subjected to PCR. Primers were designed from the nucleotide sequence of a bullfrog CE cDNA (*29*) as follows: forward primer, 5′-TCGTAACCCTTGTTTATGGC-3′, and backward primer, 5′-TGGTTCTGCACCAATAT-ACC-3′. A 573 base-long cDNA fragment was obtained on the PCR. Then, RACE-PCR was performed using the cDNA as a PCR template. The primers for 5′ and 3′ RACE-PCR were designed from the nucleotide sequence of the 573 base-long cDNA fragment. We obtained two CE cDNA clones, and named them CE1 and CE2.

Table 1. **Summary of purification of cathepsin E.**

	Protein (mg)	Activity (unit)	Specific activity (unit/mg protein)	Yield (%)
Crude extract	13.2	328	24.7	100
Q-Sepharose column chromatography	1.92	251	482	76.6
Mono-Q column chromatography	0.06	41.1	684	12.5

Northern Blot Analysis—Digoxigenine-labeld probes for CE1 and CE2 were synthesized by PCR using a PCR DIG Probe Synthesis kit (Roche, Mannheim, Germany). Each probe was constructed from 3′-UTR of CE1 or CE2. Equal amounts of total RNA were electrophoresed on a 1% formaldehyde-agarose gel, transferred to a nylon membrane filter, Hybond-N (Amersham, Little Chalfont), and then hybridized with a digoxigenine-labeled PCR probe in DIG Easy Hyb (Roche) for 15 h at 37°C. After hybridization, the filter was washed twice with 2× SSC–0.1% SDS for 5 min at room temperature, and then twice with 0.1×SSC–0.1% SDS for 15 min at 50°C. The filter was incubated with 0.2% blocking reagent and 0.1% Tween 20 in PBS for 30 min at room temperature, and then with 1/5,000-diluted alkaline phosphatase–conjugated anti-digoxigenine antibodies in the same buffer for 1 h. It was further incubated with 1% CSPD in 0.1% diethanolamine, and 1mM $MgCl_2$ for 5 min, and then exposed to a scientific imaging film (Kodak, New York) in the dark.

In Situ Hybridization—Sense or antisense DIG-labeled RNA probes for CE and PgC were synthesized using a DIG RNA labeling kit (Roche). *In situ* hybridization was performed according to the method previously described (*30*, *31*) with a slight modification. Sections of 8 μm-thick were treated with 0.3% Triton X-100 and 0.2 N HCl, and then hybridized for 16 h at 42°C with an RNA probe (1 μg/ml for CE and 0.125 μg/ml for PgC) in a hybridization solution consisting of 10% dextran sulfate, 50% formamide, 1 mg/ml tRNA, 2.5× Denhard, 0.3 M NaCl, 2.5 mM EDTA and 20 mM Tris-HCl (pH 8.0). After hybridization, the sections were treated with 20 ng/ml heated RNase at 37°C for 30 min to remove excess unhybridized probes. Then, immunological detection of the hybridized probes in the sections was performed according to the manufacturer's instructions (DIG-probe Detection kit, Roche). The DIG-labeled RNA probe used in this *in situ* hybridization could not discriminate CE1 mRNA from CE2 mRNA.

Molecular Phylogenetic Analysis of Aspartic Proteinases—We obtained sequence data for the aspartic proteinases of various animals from the DDBJ/EMBL/Gen-

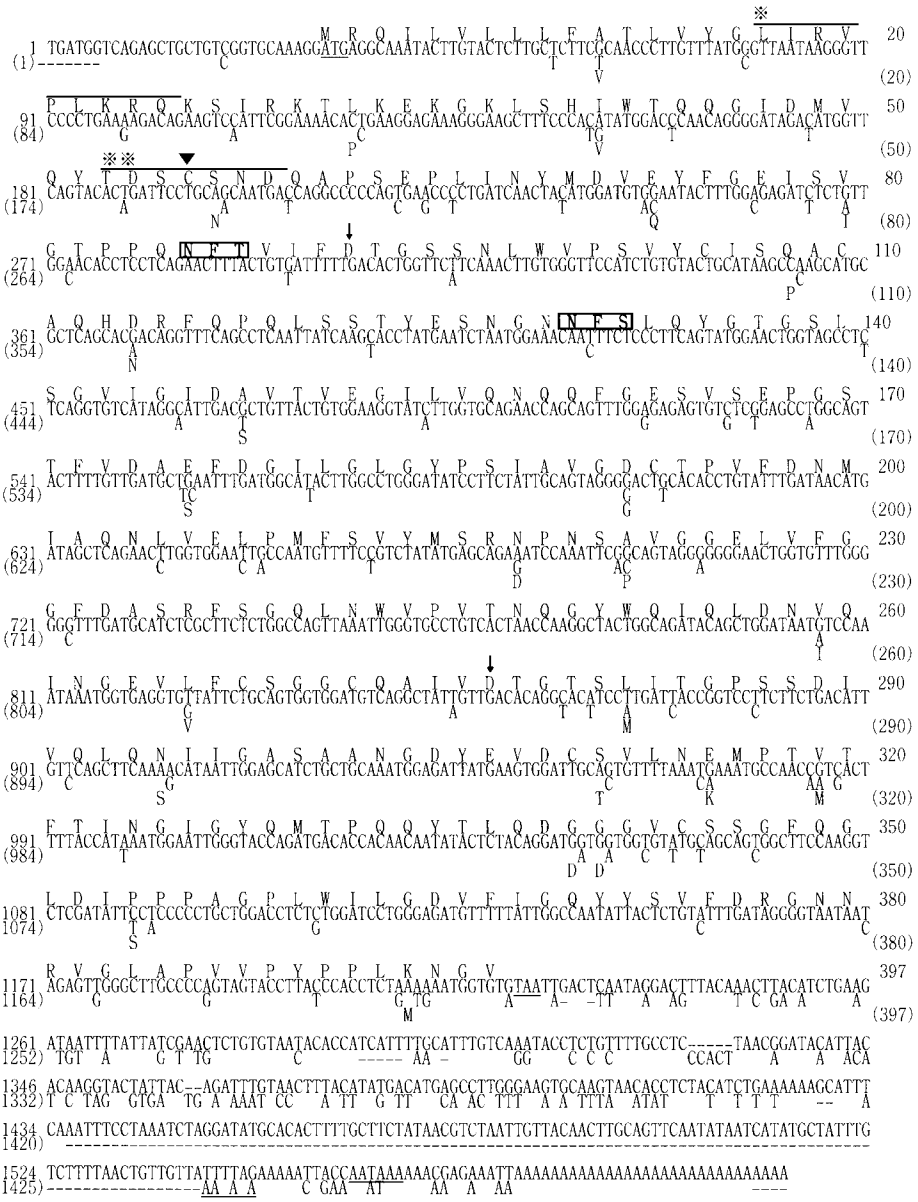

Fig. 4. **Nucleotide sequences of cDNAs for cathepsin E1 and E2, and amino acid sequences deduced from them.** The top and 2nd lines represent the predicted amino acid sequence and nucleotide sequence for CE1, respectively, while the 3rd and 4th lines represent the nucleotide sequence and predicted amino acid sequence for CE2, respectively. In the sequences of CE2, only residues differing from those of CE1 are shown. ATG, TAA and AATAAA underlined indicate the initiation codon, stop codon and polyadenylation signal, respectively. The asterisked upper line denotes the amino acids determined on Edman degradation of proCE, while the double-asterisked upper line is mature CE. Aspartic acid residues indicated by arrows (Asp^{92} and Asp^{277} in both CE1 and CE2) are highly conservative in CEs of other animals, and are putative catalytic sites. The cysteine residue indicated by an arrowhead (Cys^{56} in both CE1 and CE2) is also highly conserved, and is probably involved in inter-molecular disulfide bonding. The Asn residues in the sequences, Asn^{86}-Phe^{87}-Thr^{88} and Asn^{130}-Phe^{131}-Ser^{132}, enclosed by boxes are potential *N*-glycosylation sites.

Bank database. The amino acid sequences of proenzymes or mature enzymes were aligned using the program CLUSTAL W version 1.7 (*32*). Distances were estimated based on amino acid substitutions per site (p-distance, *33*). A tree was constructed according to the neighbor-joining method in the program MEGA2 version 2.1 (*34*) using *C. elegans* pepsinogen as the outgroup.

RESULTS

Purification of Cathepsin E from Tadpole Foregut—For purification of cathepsin E, we used the foregut, *i.e.* the area from the esophagus to the bile duct opening, of the digestive tract of *X. laevis* tadpoles (Fig. 1). The elution profile on Q-Sepharose column chromatography is shown in Fig. 2, and the purification steps are summarized in Table 1. In addition, a zymogram and the SDS-PAGE pattern of the purified CE are shown in Fig. 3.

The molecular masses of the purified CE estimated by SDS-PAGE under reducing and non-reducing conditions were 42.5 kDa and 76 kDa, respectively. Therefore, CE is suggested to be a dimeric form. During purification, the CE band shifted toward the anodic position on zymography. The molecular size of the purified CE treated with acid (pH 2.4, 20°C, 20 min) was the same as that not treated with acid (Fig. 3B), suggesting that the purified CE is a mature form. As described later, the partial amino acid sequence of the N-terminal region of CE was identical to the corresponding sequence of a mature enzyme predicted from CE1 cDNA (Fig. 4). Therefore, the CE purified in the present study was a mature dimeric form of CE1.

Nucleotide Sequences of Two cDNAs for Xenopus CE—Two cDNA clones for CE were isolated from adult stomach, and named CE1 and CE2. The nucleotide sequences of CE1 and CE2 were 1,604 bp long and 1,485 bp long, respectively. The two sequences contained the same length ORF encoding 397 amino acid residues, and the ORFs were highly similar to each other in nucleotides (identity, 92.9%). However, the cDNAs were different in

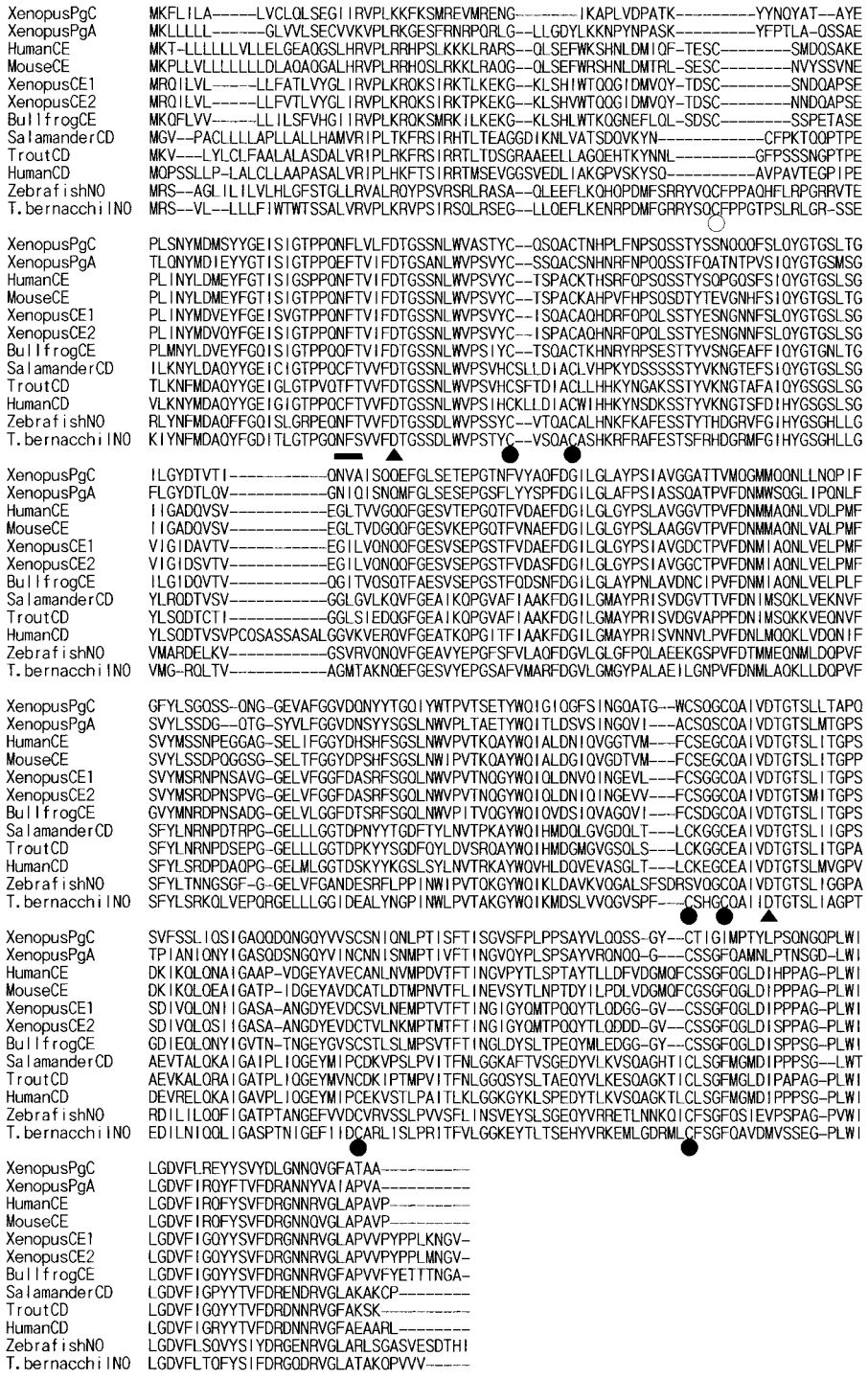

Fig. 5. **Comparison of the amino acid sequences of some aspartic proteinases.** Amino acid sequences were deduced from cDNAs for mouse CE, man CE, bullfrog CE, *Xenopus* PgC and *Xenopus* PgA, cathepsin D (CD) of man, salamander CD, trout CD, nothepsin (NO) of *T. bernacchi*, and zebrafish NO. These cDNA clones are the same as those indicated in Fig. 9. The arrowhead indicates an aspartic acid residue as a potential catalytic site. Underlining indicates a potential *N*-glycosylation site for cathepsin E. Filled and open circles indicate a cysteine residues probably involved in intra-molecular disulfide bonding and inter-molecular disulfide bonding, respectively. The latter cysteine residue in nothepsin is also found in cathepsin E.

length and sequence of the 3′-untranslated region (Fig. 4). The amino acid sequences predicted from the two cDNAs showed that 24 amino acid substitutions were dispersed throughout their entire sequences (Fig. 4), suggesting that the two CEs are encoded by two distinct genes, as discussed later. Each sequence had two aspartic acid residues as catalytic sites (Asp^{92} and Asp^{277}), which are highly conserved in all aspartic proteinases. Six cysteine residues (Cys^{105}, Cys^{110}, Cys^{268}, Cys^{272}, Cys^{310}, and Cys^{344}) and another cysteine residue (Cys^{56}) were also conserved in the *Xenopus* CEs (Fig. 4). The former six residues are presumably responsible for intra-molecular disulfide bonding, and the latter probably participates in homo-dimer formation through inter-molecular disulfide bonding.

The partial amino acid sequences of purified samples were determined by Edman degradation and compared with the sequences predicted from the cDNAs. This proved that the purified CE corresponded to the CE1 cDNA (Fig. 4). The signal peptide of CE1 comprised Met^{1} to Gly^{16}, its activation peptide Leu^{17} to Tyr^{52}, and its mature enzyme portion started from Thr^{53} (Fig. 4). We

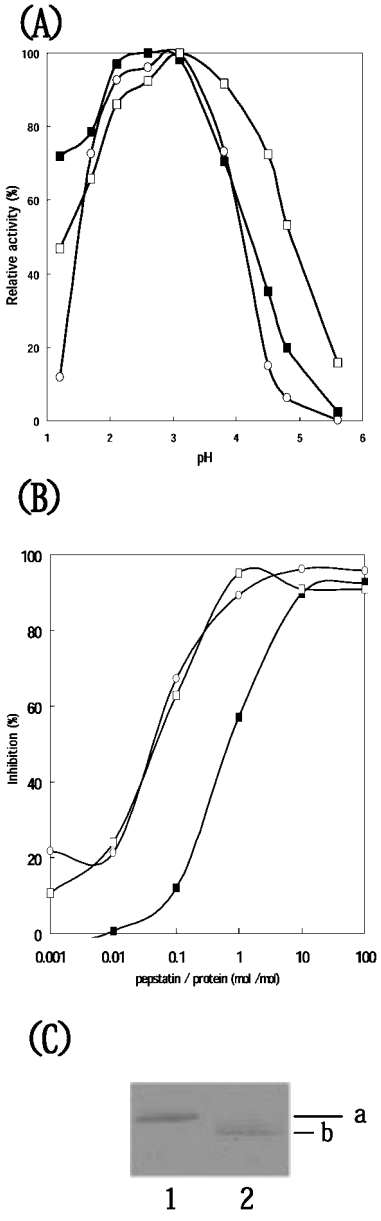

Fig. 6. **Enzymatic properties of purified cathepsin E.** A: pH dependence of the proteolytic activity of the purified CE (0.5 μg), pepsinogen A (PgA, 1 μg), or pepsinogen C (PgC, 0.5 μg) was assayed according to the method of Ikuzawa *et al.* (*27*). PgA and PgC previously purified from *Xenopus* stomach (*27*) were used as references. open circies, CE; solid squares, pepsin C; open squares, pepsin A. B: Inhibitory effect of pepstatin on CE (0.5 μg), PgC (1 μg), or PgA (1 μg) was assayed according to the method of Ikuzawa *et al.* (*27*). The inhibition was expressed as a percentage of the highest inhibition. open circles, CE; solid squares, pepsin C; open squares, pepsin A. C: Digestion of CE with Endoglycosidase H (Endo H). Endo H was added to CE (0.5 μg), followed by incubation for 15 h at 37°C. Lane 1, without Endo H-treatment (band a); lane 2, 0.5 units of Endo H (band b). After SDS-PAGE had been performed under reducing conditions, the gel was stained according to the silver staining method.

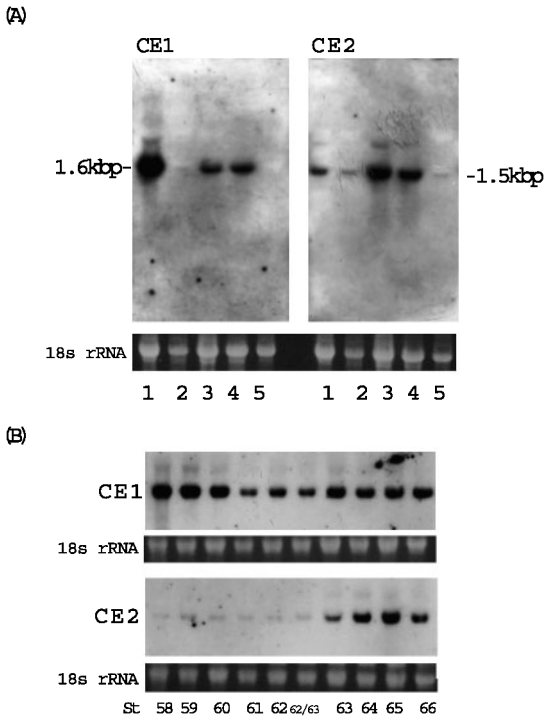

Fig. 7. **Northern blot analysis.** A: Expression of cathepsin E mRNA in some regions of tadpole and adult digestive tract. Lane 1, tadpole foregut; lane 2, adult esophagus; lane 3, anterior part of adult stomach (gastric gland region); lane 4, posterior part of adult stomach (non-glandular region); lane 5, adult small intestine including duodenum. Ten micrograms of total RNA was applied to each lane, blotted and hybridized with CE1 or CE2 probes. B: Changes in the expression of cathepsin E mRNA. Five micrograms of total RNA prepared from foregut or from the corresponding region of adult type stomach was applied to each lane. Numbers below the panels are the developmental stages defined according to Nieukoop and Faber (*25*).

predicted the cleavage site of the activation peptide of CE2 based on the sequence similarity. The results suggest that the molecular weights of the pro-enzymes of CE1 and CE2 are 41,074 and 41,140, respectively, while those of mature CE1 and CE2 are 36,771 and 36,867, respectively. The amino acid sequences of CE1 and CE2 were compared with those of aspartic proteinases (Fig. 5).

Enzymatic Properties of Xenopus CEs—Figure 6A shows the pH dependence of the proteolytic activity of *Xenopus* CE1. The highest activity was observed around pH 3.0, and the activity was undetectable at pHs higher than 5.0. Pepstatin is well known as an inhibitor of various aspartic proteinases. As shown in Fig. 6B, the activity of *Xenopus* CE was completely inhibited by equimolar pepstatin. The susceptibility of the CE to pepstatin was the same as that of PgA, and higher than that of PgC. Such properties of *Xenopus* CE were similar to those of bullfrog CE (*21*), guinea pig CE (*35*), and rabbit CE (*13*).

It is well known that a CE molecule is *N*-linked with a high-mannose type oligosaccharide chain or a complex type oligosaccharide chain. Examples of former are the CEs of human and rat gastric mucosa (*7*, *8*), and rat spleen (*36*), ones of the latter are the CEs of human and rat erythrocyte membranes (*5*), and rat microglia (*6*). Prediction of the amino acid sequence from cDNA suggested that *Xenopus* CE had two potential *N*-glycosylation sites, Asn^{86}-Phe^{87}-Thr^{88} and Asn^{130}-Phe^{131}-Ser^{132} (Fig. 4). As shown in Fig. 6C, the molecular size of the purified CE1 treated with Endo H was reduced by about 1.5 kDa.

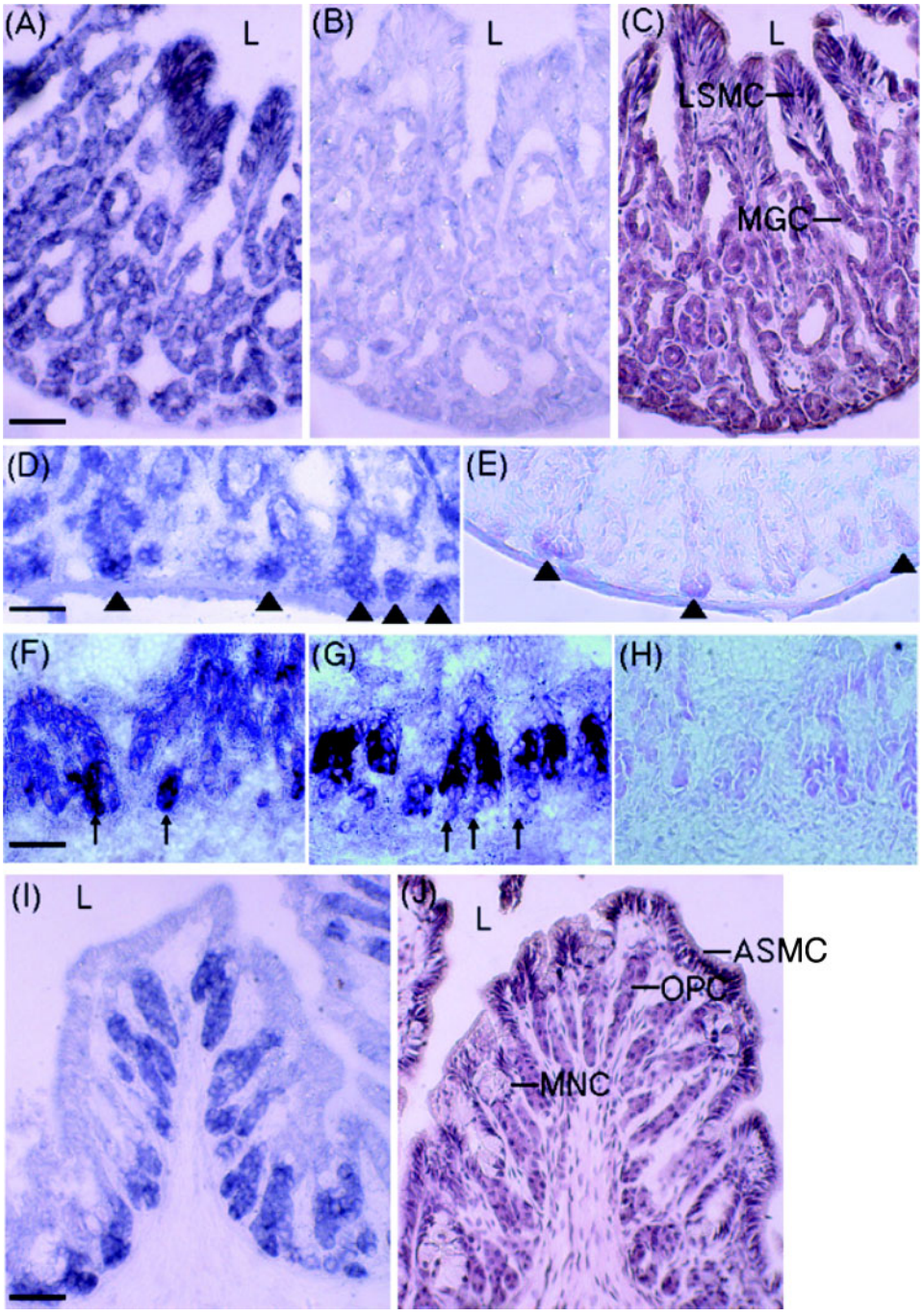

Fig. 8. ***In situ* hybridization of cathepsin E mRNA.** Cross sections of the stomach region at various stages of metamorphosis were hybridized with an anti-sense probe for CE mRNA (A, D, F, and I), an anti-sense probe for PgC mRNA (G), or a sense probe for CE mRNA (B). In addition, they were stained with hematoxylin-eosin (C and J) or methyl green-pyronin Y (E and H). A, B and C, St56 (pro-metamorphosis); D and E, St60 (early phase of metamorphic climax); F, G and H, St62 (middle phase of metamorphic climax); I and J, St65 (post-metamorphosis). The arrowhead indicates so-called "adult epithelial primordia." Arrows indicate adult epithelial cells not expressing or the expressing less PgC mRNA. L, lumen of digestive tract; LSMC, larval surface mucous cell; MGC, manicotto gland cell; ASMC, adult surface mucous cell; MNC, mucous neck cell; OPC oxynticopeptic cell. Scale bar, 50 μm.

Therefore, *Xenopus* CE is suggested to have high-mannose type oligosaccharide chain. In the present study, we could not determine whether *Xenopus* CE had one or two oligosaccharide chains at its two potential sites.

Differential Expression of CE1 and CE2 during Metamorphosis—As shown in Fig. 7A, Northern blot analysis revealed that CE1 mRNA was predominantly expressed in the foregut of tadpoles at pro-metamorphic stages St56–57. The expression level of CE2 mRNA was much lower. In frog, both CE mRNAs were expressed mainly in the anterior part (gastric gland region) and posterior part (non-glandular region) of the stomach. The expression level of CE1 was somewhat lower than that of CE2.

We examined metamorphosis-associated changes in the expression of CE mRNAs. As shown in Fig. 7B, CE1 was highly expressed in the foregut of pro-metamorphic tadpoles (St58), transiently decreased in amount during metamorphosis (St 61–62), and re-increased in amount in the stomach of froglets (after St63). Thus, the expression level was lowest at the metamorphic climax (St61). CE2 was slightly expressed in the foregut until the metamorphic climax, and gradually increased in amount after St63. The temporal expression patterns during metamorphosis were characterized by an initial decrease in CE1 mRNA, and subsequent increases in both CE1 and CE2 mRNAs.

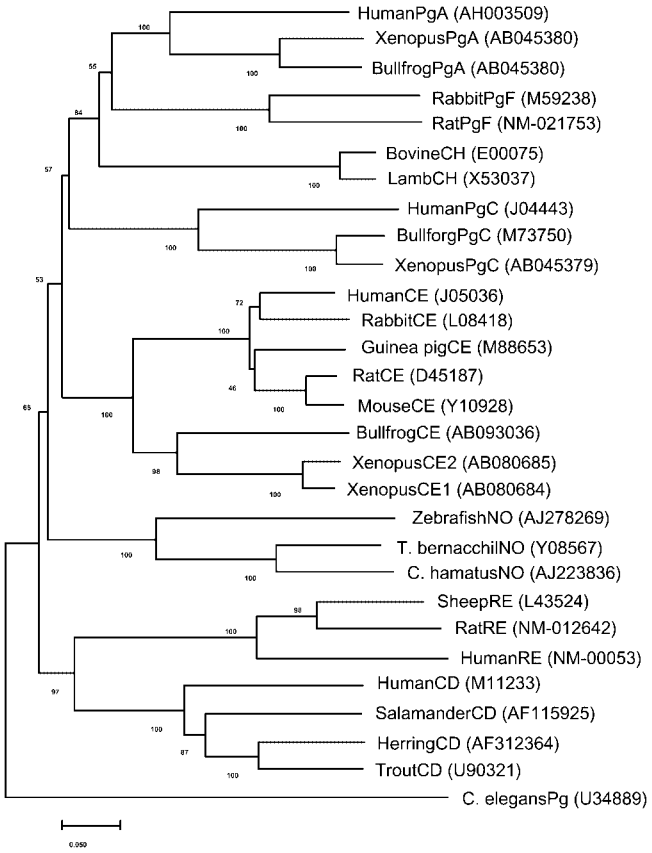

Fig. 9. **Molecular phylogenetic analysis of vertebrate aspartic proteinases.** Pepsinogen A (PgA), pepsinogen C (progastricsin, PgC), pepsinogen F (PgF), prochymosin (CH), cathepsin D (CD), cathepsin E (CE), nothepsin (NO), and renin (RE) of various animals are shown together with the accession numbers in parentheses. Each bar represents 0.05 amino acid substitutions per site, and the numbers above or below branches are the bootstrap values for 1,000 replicates. *C. elegans* Pg was used as an outgroup.

Studies involving the TUNEL method have suggested that, as metamorphosis progresses, larval epithelial cells constituting the tadpole foregut degenerate, probably due to apoptotic cell death (*24*). Therefore, the decrease in CE1 expression corresponds to metamorphosis-associated degeneration of the larval epithelium constituting the larval stomach. As described later, the increases in the expression of both CE1 and CE2 are clearly related to metamorphosis-associated formation of the adult epithelium constituting the adult stomach.

Metamorphosis-Associated Changes in Localization of CE mRNA—We examined changes in the localization of CE mRNAs in larval to adult type stomach by *in situ* hybridization. The DIG-labeled RNA probe used for the present analysis could not discriminate CE1 mRNA from CE2 mRNA.

At the pro-metamorphic stage (St56), CE mRNA signals were detected all over the larval epithelium consisting of surface mucous cells and manicotto gland cells (Fig. 8, A, B, and C). Considering the results of Northern blot analysis, the CE mRNA found at this stage is mainly CE1 mRNA.

Immediately after St60, we found spherical cell masses or islets embedded in connective tissue adjacent to a muscle layer on the basal side of the foregut. They showed intense pyronin Y-stainability (Fig. 8E). These cell masses highly resembled the adult epithelial primordia reported by Ishizuya-Oka *et al*. (*24*) in morphology, stainability, and histological location. Relatively high expression of CE was detected in the adult epithelial primordia, as well as weak expression in pre-existing larval epithelium (Fig. 8D).

After that, the adult epithelial primordia start to extensively proliferate, to grow from the basal to luminal side of the gut, and to differentiate into adult epithelial cells. Larval type epithelium was not completely replaced by adult type epithelium. At this stage, St60/61, the CE mRNA was mainly expressed in the proliferating adult epithelial cells (Fig. 8, F and H). On the other hand, the PgC mRNA was intensely expressed in the luminal side of masses of proliferating cells (Fig 8G), and weakly in the basal side of them (arrows in Fig. 8G). These PgC mRNA-positive cells are probably differentiating oxynticopeptic cells, confirming the preceding observation (*24*).

Finally, the epithelium in the gastric glands was completely replaced by adult type epithelium. At St65, after the gastric glands had already been formed, the CE mRNA signals were observed mainly in oxynticopeptic cells, and weakly in adult surface mucous cells. However, such signals were not detected in mucous neck cells (Fig. 8, I and J).

DISCUSSION

The present study showed that cathepsin E (CE) was a major member of aspartic proteinases present in *Xenopus* tadpole foregut, and its content was higher in the tadpole foregut than in the adult stomach. In addition, zymography showed that the pro-form of CE, pro-CE, was predominantly found in the crude extract of *Xenopus* tadpole foregut, and its active form was a minor component (data not shown). Although the cause is not clear, the purification procedure involving column chromatography frequently resulted in activation of pro-CE to CE, and therefore, CE was isolated. On the other hand, we obtained pro-CE as a homogeneous protein on elecro-blotting followed by SDS-PAGE, as described under "MATERIALS AND METHODS."

In general, CE is well known to be a homo-dimer molecule. A $Cys^{43}Ser$ substitution mutant of human CE lacks the ability of homo-dimer formation (*37*). As shown under Results, analysis confirmed that the CE of *Xenopus* tadpole foregut was dimeric. Based on the predicted amino acid sequence, we assume that its Cys^{56} is involved in the homo-dimer formation.

The gastric CEs of many animals excluding rat are well known to have a high-mannose type oligosaccharide chain(s) (*7*, *8*, *29*, *38*). *N*-glycosylation has been suggested to play an important role in maintenance of the proper molecular folding of CE in response to changes in the environment such as temperature, pH and redox state (*39*). We assume that the high-mannose type oligo-saccharide chain(s) found in *Xenopus* CE also have such a function.

The enzymatic properties of *Xenopus* CE, such as susceptibility to pepstatin and pH dependence of proteolytic

activity, were highly similar to those of the CEs previously investigated (*13*, *21*, *35*).

Phylogenetic Relationship between Xenopus CE and Other Aspartic Proteinases: Recently, a novel aspartic proteinase, nothepsin (NO), was identified in fish liver (*40*, *41*). Like CE, NO has some consensus motifs such as cysteine residues presumably involved in inter-molecular dimerization and potential N-glycosylation sites, while CD has no such motifs (Fig. 5). Such similarity led us to the concept that NO is more closely related to CE than other aspartic proteinases. However, molecular phylogenetic analysis suggested that NO was paraphyletic as to CE, and more similar to CD than CE (*41*).

CE had only been found in mammals until we isolated it from amphibians, *R. catesbeiana* (*21*) and *X. laevis* (the present study). The possibility arises that CE in lower vertebrates such as amphibians is more similar to fish NOs than mammalian CEs. However, the phylogenetic tree constructed by amino acid-based analysis using the neighbor-joining (NJ) method showed that the mammalian CEs and fish NOs can be classified into separate clades, and the amphibian CEs were included in the mammalian CE group (Fig. 9). Similar results were obtained with other methods such as the maximum parsimony (MP) and maximum likelihood (ML) methods. In addition, nucleotide-based analyses involving the NJ and MP method are essentially the same results (data not shown). Moreover, NO has been identified as a female- and liver-specific enzyme (*41*, *42*), while amphibian CEs, like mammalian CEs, were obtained from stomach, *i.e.* were not liver-specific enzymes. Therefore, it is reasonable to conclude that amphibian CEs belong to the mammalian CE group.

Heterogeneity of *Xenopus* Cathepsin E: The occurrence of CE isoforms has been reported in mammals. In man, one major and one minor CE have been found in stomach, and the two isoforms are identical to each other in amino acid sequence except for a 3 amino acid deletion in the N-terminal region of the major CE (*38*). Three RNA transcripts have been detected on Northern blot analysis of a human gastric adenocarcinoma cell line (*43*). Further analysis revealed that the human CE gene is a single copy one. Formation of the three RNA transcripts has been suggested to be ascribable to alternative splicing and/or alternative polyadenylation (*44*). Another example like this is well known. Two isoforms of CE mRNA have been found in rat spleen. The nucleotide sequences for the two cDNAs are essentially identical to each other except for a 33 amino acid deletion in one of them. The results strongly confirmed the participation of alternative splicing in the occurrence of the isoforms (*36*). Thus, preceding reports suggested that a molecular difference in the CE isoforms resulted from alternative splicing and/or alternative polyadenylation from a single copy gene. On the other hand, the difference between *Xenopus* CE1 and CE2 is ascribable to amino acid substitutions dispersed all over the sequence. In addition, CE1 and CE2 are differentially expressed in metamorphosing stomach. Like GATA-1 genes (*45*), the genome of *Xenopus laevis* probably has two distinct genes for CE, and the two genes were probably formed through gene duplication. The *Xenopus laevis* genome is considered to be tetraploid from an evolutionary viewpoint. The two *Xenopus* CE genes, like GATA-1, are differentially regulated.

In the present study, we focused our attention on one aspartic proteinase, cathepsin E, and investigated its expression and tissue localization during metamorphosis-associated remodeling of larval to adult type epithelium in stomach. Studies on remodeling have been extensively performed using the small intestine of anurans (*26*, *31*, *46–48*). During metamorphosis, larval type epithelial cells of the small intestine are well known to degenerate, and are replaced by adult type epithelium. The adult cells originate from so-called ″adult epithelial primordia.″ Recently, it was suggested that, at the metamorphic climax, a high level of Shh expression is detected in the primordia (*47*). The Shh expression was transiently up-regulated by a thyroid hormone (TH) signal (*48*). BMP-4 was also highly expressed in connective tissue surrounding the primordia. Shh and BMP-4 are considered to be essential factors involved in the epithelial-mesenchymal interaction during the remodeling of small intestine (*31*, *47*), and these key molecules are probably activated by the TH signaling. This model may be applicable to the remodeling of the stomach.

In the near future, we will isolate and characterize key molecules responsible for the stomach remodeling by mean of subtraction analysis, and investigate the roles of the molecules in the adult epithelial primordia of stomach.

The nucleotide sequence data reported in the present paper will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases with accession numbers AB080684 and AB080685.

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