cimp1, A Novel Astacin Family Metalloproteinase Gene from East African Cichlids, Is Differentially Expressed Between Species During Growth

Teiya Kijimoto,* Masakatsu Watanabe,* Koji Fujimura,* Masumi Nakazawa,† Yasunori Murakami,‡ Shigeru Kuratani,† Yuji Kohara,‡ Takashi Gojobori,‡ and Norihiro Okada*§

*Graduate School of Bioscience and Biotechnology, Tokyo Institute of Technology, 4259-B21, Nagatsuta-cho, Midori-ku, Yokohama, 226-8501, Japan; †Laboratory for Evolutionary Morphology, Center for Developmental Biology, RIKEN Kobe, 2-2-3 Minatojima-minami, Kobe 650-0047, Japan; ‡National Institute of Genetics, Yata, 1111, Mishima, Shizuoka 411-8540, Japan; and §National Institute for Basic Biology, Okazaki, 444-8585, Japan

Lake Victoria cichlid fishes are excellent examples of explosive adaptive radiation. Although Lake Victoria cichlids are believed to have arisen during a short period (~14,000 years), they have various species-specific phenotypes. One important phenotype that distinguishes each species is the shape of the jaw, which has diverged to adapt to the wide variety of trophic habitats present in the lake. Here we demonstrate a new approach to investigate the diversification of cichlid jaw morphology at the genetic level by examining differentially expressed genes. We used a DNA chip to compare gene expression levels between closely related cichlid fishes. This analysis indicated that the expression of some genes differed in the larvae of two cichlid species. One such clone encodes a new astacin family metalloproteinase. The expression level of the isolated gene, named cimp1, was analyzed in more detail by real-time quantitative reverse transcription-polymerase chain reaction. A significant difference in cimp1 expression was observed between two Haplochromis cichlid species during development. Using in situ hybridization, we found that this gene is expressed only in head and gill epithelia. Biochemical analysis showed that cichlid metalloproteinase 1 (CIMP1) has proteolytic activity, a common attribute of all astacin family proteins. Because some astacin family proteins contribute to morphogenesis in animals, CIMP1 is expected to participate in species-specific head morphogenesis in cichlids. This is the first study to demonstrate that differentially expressed genes among cichlids can be identified using a DNA chip.

Introduction

Lake Victoria, which is situated in the East African Plateau, harbors more than 500 endemic haplochromine species (Seehausen 1996, Turner et al. 2001). Geological evidence indicates that this lake dried up and refilled 15,600–14,700 years ago (Johnson et al. 1996). Molecular phylogenetic analysis suggests that Lake Victoria cichlids have extremely low neutral genetic variation (Nagl et al. 2000, Watanabe et al. 2004). These facts indicate that Lake Victoria cichlids have diverged over a surprisingly short period. Moreover, Lake Victoria cichlids can breed fertile hybrids in unusual environments such as laboratory tanks (de Caprona 1984) or the turbid eutrophic region of the lake (Seehausen, van Alphen, and Witte 1997). These observations also suggest that the Lake Victoria haplochromine species are genetically close.

On the other hand, Haplochromis in Lake Victoria exhibit a wide variety of trophic specializations, including feeding on insects, algae, mollusks, and scales or predation on other Haplochromis (Greenwood 1974). The jaw morphologies of cichlids reflect the adaptation of the species to their trophic habitats. Indeed, Greenwood (1974) suggested that the evolutionary success of the Haplochromis species flock could be attributed to head and dentition diversity.

Thus, the Lake Victoria cichlids have evolved diverse jaw morphologies despite their close genetic relatedness. Furthermore, the anatomical differences between Lake Victoria cichlid species are maintained after two generations of breeding in the laboratory under standardized conditions (Bouton et al. 1999). These facts suggest that heritable genetic elements are responsible for their species-specific jaw morphology. However, the genetic factor(s) responsible for the distinct morphological characteristics has not been identified.

Organ morphogenesis is controlled by a variety of genes (see Carroll, Grenier, and Weatherbee 2001). It is unlikely that every Lake Victoria Haplochromis species has species-specific genes that account for their unique morphological features. It is more reasonable to expect that species-specific jaw morphologies result from the differential expression of genes that are shared among cichlid species. Hence, we focused on identifying such genes that may be involved in jaw morphogenesis.

Because the jaw is one of the most divergent of cichlid features, it is critical that we characterize species-specific expression profiles toward the goal of elucidating the molecular mechanisms of species-specific jaw morphogenesis. In this study, we introduce a new approach to investigate this issue. Because Lake Victoria cichlids are genetically similar, a DNA chip can be used to screen for genes that are responsible for cichlid jaw morphogenesis. DNA chips (microarrays) have been used to compare genome-wide gene expression levels in tissues or cells under different conditions (Gasch et al. 2000). Recent reports also show that a DNA chip can be used to analyze developmental pathways (Michaut et al. 2003) and to identify regulatory gene sets and their regulators (Segal et al. 2003). We report here the first examples of genes that are differentially expressed between two cichlid species, as screened using a DNA chip.

Materials and Methods

Cichlid Fishes

The cichlid species we used in this work were as follows: Haplochromis parvidens (HP) (although this species was
originally described by Greenwood [1959] and reclassified as Lipochromis parvidens by Greenwood [1980], here we use Haplochromis chilotes (HC), H. sp. “rockkribensis” (HK) (these two species were also reclassified as Paralabidochromis chilotes and P. sp. “rockkribensis,” respectively; see a review in Seehausen [1996] and Greenwood [1959] for HC), and H. sp. “red tail sheller” (HR); (this species was also reclassified as Ptyochromis sp. “red tail sheller,” see Greenwood [1980] and Seehausen [1996] for description of Ptyochromis). The fishes were imported through a local pet supplier and maintained under constant conditions (28°C, 12-h dark/12-h light cycle) in 40-Liter tanks. Adults were fed three times daily with commercial food. One male and three to four females were placed in a tank to form pair bonds. Eggs hatched around 4–5 dpf and began to eat food 12–13 dpf, after which they were released into tanks specific for each species and fed three times daily with commercial food.

Isolation of Total RNA from Cichlids

Total RNA was purified from the whole body of HC at various growth stages using the RNasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. Purified RNA was suspended in RNase-free water, and the RNA quality was assessed by electrophoresis. RNA was stored at 4°C.

Construction of the complementary DNA Library and DNA Chips

HC complementary DNA (cDNA) was synthesized by SuperScriptII reverse transcriptase (Invitrogen, Carlsbad, Calif.). cDNA was purified and fractionated by a cDNA size-fractionation column (Invitrogen) to exclude short cDNA fragments. cDNAs (500–1,000 bp) were cloned into pGEM-T (Promega, Madison, Wisc.). A total of 1,792 clones were spotted on poly-L-lysine–coated glass slides according to Nakazawa et al. (2003).

Relative messenger RNA (mRNA) expression levels were compared between two cichlid species, namely HP and HR, using the DNA chip system described above. The procedure was performed in duplicate by two independent researchers. Amplification of total RNA from cichlid larvae was performed as described by Luo et al. (1999). Total RNA was isolated from the lower jaw, premaxilla, and maxilla of three larvae from each of the two cichlid species (HP and HR).

Total RNA was amplified ~400-fold. Amplified RNA (1 μg) from HR and HP was tagged with Cy3- and Cy5-labeled uridine (Amersham Bioscience, Piscataway, N.J.), respectively, by reverse transcription (RT) using random hexamer primers. Labeled target cDNA was mixed and hybridized to a single DNA chip for 14 h at 55°C. Hybridized DNA chips were scanned using a ScanArray 4000 (Perkin Elmer, Boston, Mass.). After normalization, scanned data were analyzed using QuantArray (GSI Lumonics, Nepean, Canada).

In Situ Hybridization

Full-length cimpl cDNA was cloned into pGEM-T (pGEM-CiMP1). Digoxigenin (DIG)-labeled sense and antisense RNA probes were generated from linearized pGEM-CiMP1. The plasmid construct was digested with NotI to generate the sense probe and NcoI to generate the antisense probe. Transcription was performed with DIG RNA-labeling mix (Roche, Basel, Switzerland) according to the manufacturer’s instructions.

Whole-Mount In Situ Hybridization

A series of procedures of whole-mount in situ hybridization was performed as described by Murakami et al. (2001) with the exception that the washing step (with 50% formamide, 5 × standard saline citrate [SSC], 1% sodium dodecyl sulfate [SDS]) was performed once for 30 min at 65°C. Detection of the DIG-labeled RNA probe and coloration of specimens was performed according to Murakami et al. (2001).

In Situ Hybridization in Frozen Sections

The reagents for hybridization and immunodetection of DIG-labeled probes were the same as for whole-mount in situ hybridization.

Samples stored at −20°C in methanol were rehydrated with a series of ethanol dilutions and then replaced with 30% sucrose. The samples were embedded in OCT compound (Sakura Finetek, Torrance, Calif.) and frozen at −80°C until sectioning. Sectioning (10 μm) was performed using a Leica CM1850. Sections were placed on MAS-coated glass slides (Matsunami, Osaka, Japan) and dried immediately. The glass slides were rinsed in diethylpyrocarbonate-treated water. Samples were fixed in 4% paraformaldehyde–phosphate-buffered saline (PBS) containing 0.2% glutaraldehyde. After washing the fixative with PBS, the samples were acetylated in triethanolamine-HCl to reduce nonspecific hybridization of the probe. Prehybridization was performed at room temperature (rt), and hybridization was performed at 60°C. The sections were hybridized with the same probes used for the whole-mount experiments. The specimens were washed with 2 × SSC for 5 min at 60°C, 0.2 × SSC for 1 h at 65°C, and gradually cooled from 65°C to rt in 0.2 × SSC. Samples were soaked in 0.2 × SSC for 5 min at rt and equilibrated with NT buffer (0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl) for 5 min at rt. The sections were incubated with 1% blocking solution (Roche) in NT buffer for 1 h at rt. For immunodetection of DIG-labeled probes, samples were incubated with alkaline phosphatase–conjugated anti-DIG Fab fragments (diluted 1:4,000; Roche) in blocking solution. Bound antibodies were washed with NT buffer three times for 5 min at rt. Alkaline phosphatase was activated in NTM buffer (0.1 M Tris-HCl, pH 9.5, 0.1 M NaCl, 50 mM MgCl2) for 10 min at rt. Color development was accomplished with 5-bromo-4-chloro-3-indolyl phosphate (BCIP)/nitro tetrazolium blue chloride (NBT) (Sigma, St. Louis, Mo.) in NTM buffer according to the manufacturer’s instructions.
Quantitative Real-Time RT–Polymerase Chain Reaction

Quantitative real-time polymerase chain reaction (PCR) was performed using the Quantitative SYBR Green RT-PCR kit (Qiagen) with 40 ng total RNA from three cichlid larvae according to the manufacturer’s instructions. Amplification and detection of products was performed using an iCycler iQ real-time PCR analysis system (Bio-Rad, Hercules, Calif.). Glyceraldehyde-3-phosphate dehydrogenase was used as an internal control. Experiments were performed three times each.

Expression of cichlid metalloproteinase 1 in *Escherichia coli* Cells

The region encoding mature cichlid metalloproteinase 1 (CiMP1) (amino acids 59–256) was cloned into the pET28a expression vector (EMD Biosciences, San Diego, Calif.). To obtain recombinant protein with a C-terminal His-tag, primers were designed to amplify the mature region as follows: MP1-CF01, 5′-TATACCATGGAAAATGCTGTTCCAT-3′; MP1-CR01, 5′-ATATCTCGA GGCATCCATAAGGCTT-3′. The amplified mature region was digested with *Nco*I and *Xho*I, and the resulting fragment was cloned into pET28a. The construct was transformed into *Escherichia coli* BL21(DE3), and the culture was grown at 18°C. The cells were harvested by centrifugation for 3 h after induction by 0.4 mM isopropyl-β-D-thiogalactopyranoside and subsequently stored at −20°C.

Partial Protein Purification

Proteins were extracted by sonication of the recombinant *E. coli* in extraction buffer A (20 mM Tris-HCl, pH 8.0, 0.5 M NaCl). Most of the recombinant CiMP1 was sequestered in insoluble inclusion bodies. The insoluble fraction was suspended in buffer A and centrifuged at 40,000 × *g* for 20 min at 4°C. After centrifugation, the supernatant was incubated in 40% (v/v) Triton X-100 and centrifuged for 30 min at 4°C. The precipitate was resuspended in buffer B (20 mM Tris-HCl, pH 8.0, 0.5 M NaCl, 6 M guanidine hydrochloride). After centrifugation at 200,000 × *g* for 30 min at 4°C, the supernatant was centrifuged at 4°C for 30 min at 4°C. The precipitate was resuspended in denaturing buffer (50 mM Tris-HCl, pH 8.0, 0.5 M NaCl, 6 M guanidine hydrochloride). After centrifugation at 40,000 × *g* for 30 min at 4°C, the supernatant was applied to a HiTrap–chelating HP column (5 ml, Amersham Biosciences) that had been previously charged with Ni²⁺ according to the manufacturer’s instructions. The column was washed extensively with denaturing buffer (as indicated above) and washing buffer (50 mM Tris-HCl, pH 8.0, 0.5 M NaCl, 6 M guanidine hydrochloride, 30 mM imidazole). Proteins were eluted with elution buffer (50 mM Tris-HCl, pH 8.0, 0.5 M NaCl, 6 M guanidine hydrochloride, 0.5 M imidazole).

Proteolytic Zymography

For the analysis of proteolytic activity of CiMP1, the insoluble fraction and partially purified enzyme fraction were subjected to gelatin zymography according to Hung et al. (1997). The protein-degrading activity in protein samples can be detected by the appearance of a coomassie brilliant blue (CBB)-negative band on the acrylamide gel containing its protein substrate. Part of the insoluble fraction was incubated for 30 min at 37°C in 200 μl of SDS–polyacrylamide gel electrophoresis (PAGE) sample buffer lacking β-mercaptoethanol so as to not reduce the functional disulfide bond. A Tris-Tricine SDS-PAGE system was used for electrophoresis. The gel was 7.5% acrylamide containing 0.1% gelatin. After electrophoresis, the gel was incubated twice for 1 h at 28°C with 2% (w/v) Triton X-100 in 20 mM Tris-HCl (pH 8.0) followed by incubation in 20 mM Tris-HCl (pH 8.0) containing 0.1 mM ZnCl₂ (renaturation buffer) for 16 h at 28°C. The gel was stained with Quick-CBB (Wako, Osaka, Japan). When we tested other proteins such as fibronectin, reduced and carboxymethylated bovine serum albumin (BSA), casein, and collagen I for zymography, the concentration of proteins in the gel was adjusted to 0.5%–1% (w/v) depending on the samples. For testing the effect of ethylenediaminetetraacetic acid (EDTA), an aliquot of 0.5 M EDTA was added to the renaturation buffer to give a final concentration of 20 mM. The gel was incubated in the buffer for 16 h at 28°C and stained with Quick-CBB.

Results

Screening for Genes that are Differentially Expressed Between Two Cichlid Species

As mentioned in *Materials and Methods*, this work primarily used the cichlid species HP, HK, and HR. We focused on gene expression levels that may affect jaw morphology and used a DNA chip to screen the genes that are differentially expressed between the Lake Victoria haplochromine species HP and HR. In preliminary studies, we examined the morphology of cichlid larvae heads at various ages by staining the cartilage with Alcian blue and the bone with Alizarin red. We monitored the head morphology around 20 dpf, but we could not find obvious differences in the characteristics between the two species before that time. However, mineralization of bones in the head is drastically achieved from 10 to 20 dpf, and for all the fishes we tested for staining at 20 dpf, most bones in the head were stained with Alizarin red. Head morphology became distinct around 50–60 dpf in each species. We therefore compared gene expression levels between HP and HR using 20 dpf larvae, because these species display a prominent difference in lower jaw length during adulthood.

We screened for clones for which the difference in fluorescence intensity was more than fourfold between the two species. This criterion defined “differentially expressed genes” in this study. We repeated the same procedure three times for the same amplified RNA samples of jaws. Furthermore, two investigators in this laboratory used different amplified RNA samples for three comparisons of expression levels between HP and HR, as described in *Materials and Methods*. Three clones were identified reproducibly in these separate experiments. The isolated fragments were as follows: clone no 08f04, an astacin family–like gene, the signal for which was approximately sixfold stronger in HR than HP; clone no 02a11, similar to...
mouse c-type lectin; and clone no 34e11, similar to epithe-
lin-granulin. We used clone no 08f04 in subsequent anal-
yses because some astacin family proteins are responsible
for bone morphogenesis in other animals (Wardle, Welch,
and Dale 1999; see also Discussion)

Sequence Analysis of CiMP1

The sequence of the full-length clone no 08f04 was
obtained from our expressed sequence tag data (Watanabe
et al. 2004). We used RT-PCR to isolate this sequence from
cDNA libraries for HC, HP, HK, and HR. Both interspecies
variation and intraspecies polymorphisms were observed,
as often occur in analyses of Lake Victoria cichlids. The
sequence identity of cimp1 among species is more than
98% (data not shown). The open reading frame (ORF)
for clone no 08f04, which we named cimp1 (DNA Data
Bank of Japan accession number AB192347), was 768
bp encoding 256 amino acids. A BlastX database search
suggested that this gene is highly similar to carp nephrosin,
which was originally purified from the head kidney of carp
and identified as a member of the astacin family (Hung et al.
1997).
cimp1 is also similar to medaka hatching enzyme
that involves two genes, high choriolytic enzyme (HCE)
and low choriolytic enzyme (LCE, fig. 1). According to
GenBank, the other known astacin family proteins are as
follows: astacin, a digestive enzyme from crayfish (Stocker,
Sauer, and Zwilling 1991); meprin, a multiple domain
membrane component comprised of homologous alpha
and beta chains (Jiang et al. 1992); bone morphogenetic
protein-1 (BMP1), which is involved in vertebrate morpho-
genesis (Dumermuth et al. 1991); tolloid from Drosophila
(Shimell et al. 1991); and hydra metalloproteinase 1
(HMP1) and HMP2 from hydra (Yan et al. 1995, 2000).
Members of this protein family have an N-terminal signal
and propeptide for secretion from cells and correct activa-
tion in tissues, respectively. The mature proteins require
a zinc ion for their activity and thus contain an astacin sig-
nature motif (HEXXHXXGFXHEXXRXDRD) that serves
as a zinc ion–binding site and proteinase catalytic center.
These proteins also contain a met-turn (SXMHY). The
cimp1 sequence encodes all these domains (fig. 1). In some
members of this protein family, the C-terminal end of the
proteinase domain has an additional domain, such as an epi-
dermal growth factor-like motif and/or a CUB (complement
components C1r-C1s, the sea urchin protein Uegf, and
BMP1) motif that is not present in CiMP1.

We identified the mature region from sequence simi-
larity with other genes (fig. 1). The molecular weights for
the full-length and mature protein were predicted to be ~33
dkDa and ~22 kDa, respectively. The putative signal peptide
was determined by the hydrophobicity ratio of amino acid
residues. No sequence similarity to the putative signal or
propeptide was found in GenBank.

Expression Pattern Analysis of cimp1

In Situ Hybridization

We performed whole-mount in situ hybridization at the
stage of 20 dpf larvae, which was used for comparison of
gene expression on the DNA chip. Using cimp1 antisense
probes generated from HR, HP, and HK, we detected cimp1
mRNA only around the head in all these species (figs. 2 and
3), whereas the sense probe did not generate a significant
signal (data not shown). The mRNA expression was strongly
detected in HR; however, the signal of HP was so weak. For
example, HR larvae indicate strong hybridization signals on the lateral line (supraorbital canal) and part of premaxilla, both of which are membranous bones, and on epithelia of chin and gills, where HP indicated very weak signals except for around the premaxilla (fig. 2). Next we examined expression of cimp1 at various time points of growth by using HK larvae. Results showed that cimp1 appeared just after hatch and keep their high expression level through the growth stage that we tested (fig. 3). Although the extent of the difference of the signal intensity of in situ hybridization is not necessarily consistent with the data of DNA chips, the signal is apparently stronger in HR than in HP.
We also performed in situ hybridization on frozen sections of HK at 10 dpf for a more detailed profile of cimp1 expression. A clear hybridization signal was observed in the epithelial region of the specimens but not around bones (fig. 4). Hybridization to sections of body parts, including kidney, did not produce a signal (data not shown), consistent with data from the whole-mount in situ hybridization study.

**Quantitative RT-PCR**

We performed quantitative real-time RT-PCR (fig. 5) to examine cimp1 expression during larval growth and compared the levels of cimp1 expression in different cichlid species. We had difficulty obtaining sufficient HR larvae after DNA chip experiments due to the aggressive behavior of the adults, which precluded consistent breeding. Like HR, HK has a shorter lower jaw than HP (see the detailed description for fish morphology in Discussion). Thus, HK larvae were used instead of HR, and expression levels of cimp1 were compared with those of HP in this study. Relative cimp1 expression was analyzed at different growth stages for each species. Maximum expression was observed at 10 dpf in HK and at 15 dpf in HP. HK had higher levels of gene expression than HP in most of the time points we tested.

**Analysis of the Biological Role of CiMP1**

**Relationship with Other Astacin Family Proteins**

To better understand the biological role of CiMP1, we searched the GenBank database for astacin family proteins having conserved amino acid sequences. We aligned the deduced CiMP1 sequence with astacin family proteins such as the medaka (Oryzias latipes) hatching enzymes, known as HCE and LCE, carp (Cyprinus carpio) and zebrafish (Danio rerio) nephrosin, hydra (Hydra vulgaris) HMP1,
human BMP1, mouse meprin1a, three unknown Fugu (Fugu rubripes) sequences, two unknown zebrafish sequences, and crayfish (Astacus astacus) astacin. As performed in a previous study by Hung et al. (1997), we aligned all of the astacin domains (~200 amino acids) using the ClustalX sequence alignment program (Thompson et al. 1997). A phylogenetic tree was subsequently constructed using these same sequences. A Neighbor-Joining tree (p-distance method with complete deletion) generated by MEGA2 (Kumar et al. 2001) showed that cimp1 and the nephrosin gene are clearly distinguishable but are included in a sister group (fig. 6). cimp1 also was distinguishable from the hatching enzymes, suggesting another possible function for this gene. These results were supported by high bootstrap values (fig. 6).

**Enzymatic Activity of Recombinant CIMP1**

Because our sequence analysis suggested that cimp1 is a member of the astacin family of metalloproteinases, the protein product was predicted to have the ability to degrade extracellular matrix (ECM) proteins and to require a zinc ion for activity. Hence, we tested whether recombinant CIMP1 had proteolytic activity in vitro using a zymography assay. The putative mature protein region of cimp1 cDNA was cloned into pET28a, and CIMP1 was expressed in bacteria. A partially purified fraction containing recombinant CIMP1 was subjected to gelatin zymography. As shown in figure 7, recombinant CIMP1 had gelatin-degrading activity. When the enzyme solution was treated with SDS-PAGE sample buffer including reducing reagent, no proteolytic band was observed (data not shown), suggesting that the activity requires a disulfide bond. Inclusion of 20 mM EDTA in the reaction (see Materials and Methods) reduced the gelatin-degrading activity of CIMP1 (fig. 7), supporting the prediction that zinc is required for enzymatic activity (as with other astacin family proteases). Other potential substrates of CIMP1 were also tested by zymography. Recombinant CIMP1 degraded not only gelatin but also casein, a general substrate of many proteinases in vitro, reduced-carboxymethylated BSA, which is degraded by nephrosin, fibronectin, which is also degraded by hydra HMP1 (Yan et al. 1995), and denatured collagen I (data not shown).

**Discussion**

**DNA Chips can be Used to Identify Genes Responsible for Species-Specific Cichlid Morphogenesis**

We constructed a DNA chip to identify and to isolate genes that are differentially expressed between species. According to our expressed sequence tag (EST) analysis, cDNA sequences of the expressed genes are almost identical among species (Watanabe et al. 2004). Thus, we consider that HC cDNA used here for the construction of cDNA library and DNA chip will not affect the results that compare gene expression levels between two cichlid species that are not HC. Among the species we used, HP has the longest lower jaw (43.3%–55.5% of head length, Greenwood 1974). HK, being included in Paralabidochromis species according to a recent reclassification (Seehausen 1996), is recognized by a short head (less than 33% of...
the standard length) and a relatively long lower jaw (between 34% and 39% of the head length; Seehausen 1996). HR, being included in Ptyochromis according to recent reclassification, is mainly identified by dentition, tooth shape, and a relatively short lower jaw (22%–38% of the head length; Seehausen 1996). We expected to isolate the genes that may create such morphological difference and to testify their biological functions after isolation of the genes.

Using the DNA chip, we detected three clones that exhibited differential expression between the two cichlid species. The expression of clone no 34e11, which is similar to epithelin-granulin, was ~16-fold higher in HR compared with HP. The expression of clone no 02a11, which is similar to c-type lectin, was ~10-fold higher in HP compared with HR. Clone no 08f04, subsequently named cimp1, was expressed approximately sixfold higher in HR compared with HP and putatively belongs to a metalloproteinase family that degrades the ECM and/or other protein substrates. Because metalloproteinases that degrade the ECM often play important roles in organ development (for review see Perris and Perissinotto 2000), we first analyzed the function of CiMP1 in vitro. Nephrosin is a single-locus gene while other two genes belong to the multigene family. This was another reason to choose this gene for the analysis, because we planned to quantify the gene expression level by using real-time quantitative RT-PCR after the DNA chip experiment, and multi-loci genes are difficult to analyze their quantity because of their high similarity among paralogs. The other candidate genes identified in this study will be described elsewhere.

Analysis of CiMP1 Sequences

We compared cimp1 cDNA sequences among Lake Victoria cichlids. The signal and propeptide sequences encoded by the cDNAs were perfectly conserved among the four Haplochromis species HP, HR, HK, and HC. There is no fixed sequence difference of coding region among HP, HR, HK, and HC, implying that the enzymatic activity of CiMP1 is essentially the same among cichlid species. This suggests that the level of cimp1 expression is important for cichlid differentiation.

Astacin family proteins can be divided into subgroups as follows (Geier and Zwilling 1998): proteins having morphogenetic activity, such as BMP1; proteins that degrade various bioactive peptides, such as meprin; and proteinases that degrade the egg envelope during hatching, such as medaka LCE. Phylogenetic analysis established that cimp1 is an astacin family member (fig. 6). However, it is interesting that nephrosin and cimp1 are not included in any of the subgroups described above, at least among the amino acid sequences we considered. This indicates that nephrosin and cimp1 comprise a new subgroup in the astacin family. Hence, further analysis is needed to predict the function of CiMP1.

Analysis of cimp1 Gene Expression and CiMP1 Activity In Vitro

The biological role of vertebrate astacin family members, except BMP1, is not well understood. BMP1 is a mammalian homolog of tolloid, which activates decapentaplegic
to determine the Drosophila dorsoventral axis (Shimell et al. 1991). BMP1 cleaves chordin during bone morphogenesis. Chordin is an antagonist of BMP4, which directs bone and cartilage formation (Wardle, Welch, and Dale 1999). It is likely that some genes of the astacin family play an important role in organ morphogenesis, and thus CiMP1 is expected to function in organ tissue morphogenesis in the head.

Astacin family proteins have various expression patterns. Carp nephrosin, which is closely related to CiMP1 (fig. 1), is expressed in various tissues such as head kidney, blood, and spleen (Hung et al. 1997). On the other hand, there is another group of astacin family genes whose expression is strictly temporally and/or spatially controlled. Hydra HMP1, for example, localizes only in the ECM in the head region and is involved in development (Yan et al. 1995). HMP2 expression is localized in the foot and is involved in foot morphogenesis (Yan et al. 2000). In this work, we found that cimp1 is expressed around epithelia in the head during all developmental stages tested (3–20 dpf, figs. 2, 3, and 4). Astacin family metalloproteinase BMP1, when expressed in the skin, is responsible for assembling dermal-epidermal junctions (Burgeson and Christiano 1997). By analogy, it is likely that epithelial expression of CiMP1, a member of astacin family like BMP1, may affect cichlid jaw morphogenesis during their growth via its protein-degrading function.

The in situ hybridization results (figs. 2 and 3) suggest that the regions in which cimp1 is expressed are similar to those of hatching enzymes. Hatching enzymes are secreted from hatching gland cells, which are distributed in the gills, lower jaws, and yolk of fish embryos (Inohaya et al. 1995). Given that cimp1 is expressed in epithelial cells of the head and gills, we initially suspected that it is an ortholog of the hatching enzymes. The medaka hatching enzyme is expressed in the hatching gland of the lower jaw and around gills in prehatched embryos, and its expression drastically drops after hatching (Inohaya et al. 1995). By contrast, cimp1 is not expressed in prehatching embryo but expressed even after hatching (fig. 3); moreover, its expression level fluctuates during growth (fig. 5). Thus, we conclude that CiMP1 is neither an ortholog of nephrosin nor of hatching enzymes. The in vitro enzymatic activity (fig. 7) and amino acid sequence of CiMP1 (figs. 1 and 6) suggest that it is a new member of the astacin metalloproteinase family derived from hatching enzymes. Interestingly, nephrosin, which is included in the same subgroup of the family, is also expressed in gills like cimp1. However, cimp1 is not expressed in internal organs such as kidney or spleen. Isolation of cichlid ortholog of nephrosin will help us to understand the evolution of fish genome and astacin family.

We used quantitative real-time RT-PCR to examine cimp1 expression as a function of cichlid development (fig. 5). In the two species that were analyzed (HP and HK), we found that expression differed in both timing and magnitude. cimp1 expression reached a maximum 5 days earlier in HK (10 dpf) than in HP (15 dpf). Because every species of Lake Victoria cichlids in our lab began to consume food at 12–13 dpf, foraging-related developmental or morphogenetic events occurring at 10–15 dpf (i.e., between the time of maximum expression of cimp1 in HK and HP) may be critical for determining the unique head morphology of these two species during growth. In blowfish, transcriprional heterochrony of genes responsible for morphogenesis can contribute to morphological differences between closely related species (Skaer, Pistillo, and Dorsky 2000).
Similarly, the transcriptional heterochrony we observed in cichlids may also contribute to the morphological differences between HK and HP. Our next goal will be to identify the factor(s) that controls \textit{cimp1} transcription. This factor may be a \textit{cis} regulatory element and/or signal transduction molecules that act upstream during development. We recently constructed a bacterial artificial chromosome (BAC) library for HC (Watanabe et al. 2003), which will help us to efficiently isolate the upstream regions of genes.

Sequence similarities between CI\textit{MP}1 and other proteins of the astacin family suggest that it should have gelatin-degrading activity. Indeed, recombinant CI\textit{MP}1 has proteolytic activity that is zinc dependent (fig. 7). In most cases, enzymes that degrade the ECM contribute to organ morphogenesis (Chin and Werb 1997). Consistent with this paradigm, some of the astacin family proteinases degrade ECM proteins and are involved in organogenesis. We observed that CI\textit{MP}1 has broad substrate specificity, as do other astacin family proteins such as BMP1 or meprin A and B. BMP1 degrades procollagens (Kessler et al. 1996, Li et al. 1996), prolylser oxidase (Uzel et al. 2001), and biglycan (Scott et al. 1999) in vitro. We observed that CI\textit{MP}1 can degrade denatured type I collagen and fibronectin, ECM proteins that function in cell adhesion, migration and/or differentiation. Taken together with the results of the in situ hybridization and quantitative real-time RT-PCR assays, this information suggests that the CI\textit{MP}1 expression level during cichlid growth affects the total mass of ECM proteins in the head structures, including the jaws. Thus, our current understanding suggests three possibilities for CI\textit{MP}1 function. CI\textit{MP}1 may degrade cell surface proteins, such as fibronectin, and thereby prevent cell migration and/or differentiation. Alternatively, CI\textit{MP}1 may degrade ECM proteins and affect cell-cell signaling mediated by ECM proteins during cichlid growth. (Importantly, ECM proteins interact with secreted signaling molecules such as transforming growth factor-β proteins and/or growth factors.) Reconstruction of ECM will take place during the growth of organs of cichlid larva. Thus, it is likely that changes in the timing of expression levels of ECM protein-degrading proteinases such as CI\textit{MP}1 will affect the jaw shapes among species. Finally, CI\textit{MP}1 may function analogously to BMP1 and degrade other signaling molecules directly.

In each case, bony tissues grow and represent species-specific jaw shape gradually but after embryogenesis. Interestingly, it should be noted that \textit{cimp1} was expressed in membranous bones of cichlid larvae. Premaxilla is one such bone, and the shape of this bone is sometimes used for the description of species-specific characteristics for cichlids. \textit{cimp1} was also strongly expressed in the tissues that are not directly related to the jaws such as chin or gills. However, expression of \textit{cimp1} in these tissues may affect the morphological characteristics of contiguous tissue such as lower jaw during the growth.

We are now preparing antibody to observe CI\textit{MP}1 localization in cichlids. Immunohistochemical analysis will help us to understand in detail the region where the protein is secreted and located. Furthermore, the antibody will also help us to understand protein quantity of \textit{cimp1} during the growth of cichlid.

A recent study of Lake Malawi cichlids (Albertson, Streelman, and Kocher 2003) illustrates the ongoing effort to elucidate the genetic factors responsible for morphological differences between closely related species using quantitative trait loci (QTL). On the other hand, we applied DNA chip technology to directly identify differentially expressed genes among closely related species on a genome-wide basis. This procedure also will help us to further observations of gene expression profiles during cichlid morphogenesis. Genome-wide gene-searching methods, such as QTL analysis and our method, will directly identify the genes required for species-specific morphogenesis among cichlids. To demonstrate the direct involvement of such genes in morphogenesis, it will be necessary to generate and analyze transgenic animals, the procedure of which was recently established in the study of transgenic zebrafish development (Kawakami et al. 2004). The application of this method to cichlids will enable us to directly test the contribution of candidate developmental genes during organ morphogenesis.

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Literature Cited


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