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Foot differentiation and genomic plasticity in *Hydra*: lessons from the *PPOD* gene family

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Abstract In *Hydra*, developmental processes are permanently active to maintain a simple body plan consisting of a two-layered, radially symmetrical tube with two differentiated structures, head and foot. Foot formation is a dynamic process and includes terminal differentiation of gastric epithelial cells into mucous secreting basal disc cells. A well-established marker for this highly specialized cell type is a locally expressed peroxidase (Hoffmeister et al. 1985). Based on the foot-specific peroxidase activity, the gene *PPOD1* has been identified (Hoffmeister-Ullerich et al. 2002). Unexpectedly, this approach led to the identification of a second gene, *PPOD2*, with high sequence similarity to *PPOD1* but a strikingly different expression pattern. Here, we characterize *PPOD2* in more detail and show that both genes, *PPOD1* and *PPOD2*, are members of a gene family with differential complexity and expression patterns in different *Hydra* species. At the genomic level, differences in gene number and structure within the *PPOD* gene family, even among closely related species, support a recently proposed phylogeny of the genus *Hydra* and point to unexpected genomic plasticity within closely related species of this ancient metazoan taxon.

Keywords *Hydra* · Foot · Basal disc · Differentiation · Phylogeny · Cnidaria

Introduction

Hydra has a simple body plan consisting of a two-layered, radially symmetrical tube with two differentiated structures

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along a single body axis: head and foot. The head is made up of a ring of tentacles surrounding a hypostome with a mouth opening, while the region below the budding zone, generally referred to as “foot,” consists of the peduncle and the basal disc (Campbell 1967). To maintain this pattern, developmental processes are permanently active in this basal metazoan, as the cells of the body column continuously divide and get displaced toward the extremities, namely, the head/tentacles and the foot. Here, they differentiate in a position-dependent manner (for recent review, see Bosch and Fujisawa 2001; Steele 2002; Bosch 2003; Bode 2003). Head- and foot-specific cell differentiations are the major patterning events in steady state *Hydra* since polyps reproduce mainly asexually by budding and since embryogenesis, as a result of sexual reproduction, occurs only seasonally.

Foot-specific cell differentiation affects all three cell lineages of *Hydra* and leads to changes in form and function of their derivatives. Changes in the interstitial cell lineage were observed for neurons and their precursors as they differentiate into neurosensory and neurosecretory cells, which accumulate and rearrange in a circular basal disc nerve net (Burnett and Diehl 1964). Moreover, their biochemical anatomy is also altered during foot differentiation (for recent review, see Koizumi et al. 2004). The cells of the endodermal epithelial lineage undergo minor morphological changes upon entering the basal disc as they reduce the number of their microvilli and pinocytotic vesicles (Davis 1975). The most dramatic changes are observed in the ectodermal epithelial cell lineage as observed both at the histological and the molecular level (Davis 1973; Dübel et al. 1987; Kobayakawa and Kodama 2002). Ectodermal epithelial cells enter the peduncle and are displaced toward the basal disc within 3 days (Dübel et al. 1987). On their way toward the basal disk, they get gradually determined (Kobayakawa and Kodama 2002) for terminal differentiation at the junction between the peduncle and the basal disc. Here, they get arrested in the G2 phase of their last cell cycle (Dübel et al. 1987) and differentiate from a cubic epitheliomuscular cell of the body column into a high-prismatic, mucous-secreting “glandulomuscular cell” (Davis 1973).

They contain up to six different types of secretory vesicles (Philpott et al. 1966; Davis 1973). After 5 days of high metabolic activity (Dübel et al. 1987), correlated with the local disintegration of the extracellular matrix in this region (Davis 1973), epithelial cells are finally sloughed off in the center of the basal disc.

Foot-specific cell differentiation has been described by means of light and electron microscopy as well as by two molecular markers: (1) the monoclonal antibody AE03 recognizing an unknown epitope present in a nematocyst subtype as well as in the mucous granules of the glandulomuscular cells (Amano et al. 1997; Kobayakawa and Kodama 2002) and (2) a peroxidase activity residing in this highly specialized epithelial cell type (Hoffmeister and Schaller 1985). The peroxidase assay has been widely applied in developmental studies in *Hydra* (e.g., Technau and Holstein 1996; Müller 1996; Grens et al. 1999; Harafuji et al. 2001; Zeretzke et al. 2002; Technau et al. 2003). The enzyme corresponding to the foot-specific peroxidase has been recently isolated and characterized for *Hydra vulgaris* (Zürich). Based on the activity of this protein, the gene *PPOD1* has been identified. The spatiotemporal expression of *PPOD1* corresponds to the biochemical activity of the peroxidase (Hoffmeister-Ullerich et al. 2002). Surprisingly, in the same approach, a related additional gene, named *PPOD2*, with high sequence identity to *PPOD1* but a differential expression pattern was found (Hoffmeister-Ullerich et al. 2002).

In this study, we characterize the *PPOD* gene family within the genus *Hydra* in more detail. We demonstrate that the expression of *PPOD* genes is tightly regulated during different foot formation processes. In addition, our study reveals an unexpected variability of this gene family at the genomic level, even among closely related species. Based on our observations, we propose that *PPOD* genes are suitable molecular markers to study foot-specific cell differentiation. The structure variations within the *PPOD* gene family among different *Hydra* species point to an unexpected genomic plasticity within the genus *Hydra* and support a recently proposed molecular phylogeny.

Materials and methods

Animals and lithium chloride treatment

Hydra vulgaris strain Basel, *Hydra vulgaris* strain AEP (Martin et al. 1997), *Hydra magnipapillata* strain 105, *Hydra carnea* strain L4, *Hydra oligactis*, *Hydra robusta* (*Pelmatohydra robusta*, a gift of T. Fujisawa, Mishima), *Hydra circumcincta* strain M5, and aposymbiotic *Hydra viridis* strain A99 were used. The animals were cultured according to standard procedures at 18°C. If not specified in the text, *Hydra* was fed on artemia three times a week. Lithium chloride treatment was performed as described before (Grens et al. 1996). *H. vulgaris* (Basel) polyps were incubated for 15 days in *Hydra* medium supplemented with 0.5 mM LiCl. LiCl-treated animals were daily fed followed by medium changes.

RT-PCR, RACE PCR

Reverse transcription-polymerase chain reaction (RT-PCR) with LiCl-treated and control animals was carried out as described before (Siebert et al. 2005). Tissue fractions were pooled as depicted in Fig. 1c. For amplification of gene-specific fragments, the following primer sets and cycle numbers were used: *H. vulgaris* (Basel) *PPOD1* (forward 5'-ACG TCT GGG AAA TGT TCA GC-3', reverse 5'-AAT TGA AAC TAT CCA ACT TAA ACA AAA GA-3'; 27 cycles), *H. vulgaris* *PPOD2* (forward 5'-AAG AAA AAC GAG CTA CAT TAG-3', reverse 5'-TAT TTG AGC TCG GTT AGC ACG-3'; 23 cycles). The cDNA samples were equilibrated by PCR with β -actin (forward 5'-AAG CTC TTC CCT CGA AGA ATC-3', reverse 5'-CCA AAA TAG ATC CTC CGA TCC-3'; 18 cycles) and *Glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*)-specific primers (forward 5'-GCC TTA TGA CAA CCA TTC AT-3', reverse 5'-TCA ACA ACA GAA ACA TCT GC-3'; 27 cycles). The annealing temperatures were 56°C for *actin* and *GAPDH* and 52°C for *PPOD* fragments. Sequences of primers for amplification of *PPOD* gene fragments from *H. vulgaris* (AEP) and *H. oligactis* will be sent upon request. Fragments were cloned in Vector pGEM-T (Invitrogen) prior to sequencing. Full-length cDNA sequences were obtained by 5'- and 3'-rapid amplification of cDNA ends (RACE) PCR as described before (Fedders et al. 2004) and deposited at GenBank: *H. vulgaris* (Basel) *PPOD2*-short (accession number: DQ073559), *H. magnipapillata* *PPOD1*, *PPOD2*, *PPOD2*-short, *PPOD2*-like (accession numbers: DQ072591, DQ073557, DQ073558, DQ073560), *H. vulgaris* (AEP) *PPOD1* (accession number: DQ073555), and *H. oligactis* *PPOD1* (accession number: DQ073556).

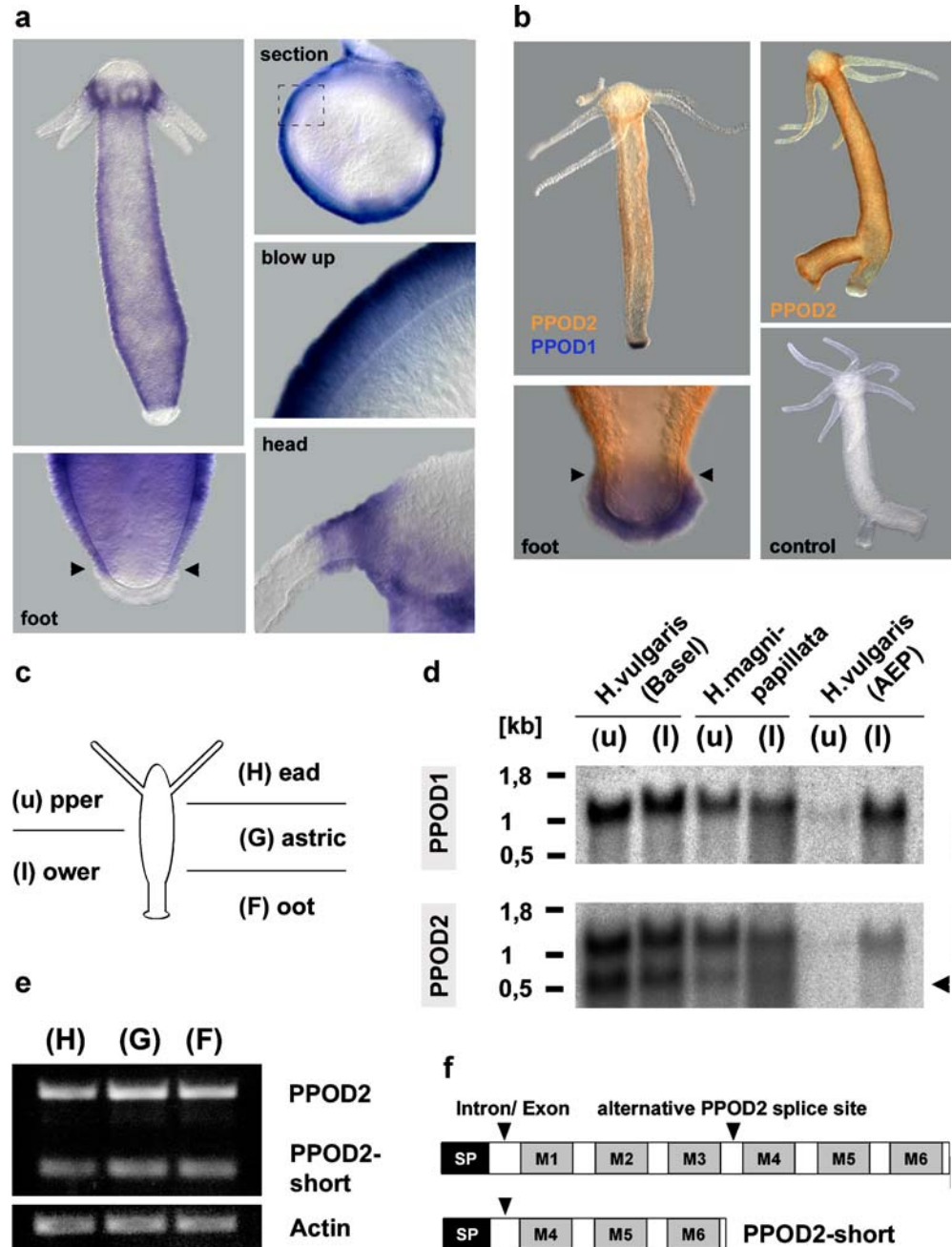
Isolation of 5'-regulatory regions of *PPOD* genes

5'-flanking regions for *PPOD* genes in *H. vulgaris* (Basel) and *H. oligactis* were isolated by a nested PCR-based approach with adapter-ligated genomic DNA fragments as described before (Devon et al. 1995; Thomsen et al. 2004; Siebert et al. 2005). Enzymes used for digestion of genomic DNA and primer sets used for amplification of specific fragments will be sent upon request to the corresponding author. Genomic sequences including the 5'-flanking regions of *H. vulgaris* (Basel) and *H. oligactis* *PPOD* genes are available at GenBank (accession numbers: DQ073561, DQ073562, DQ073563, DQ073564, DQ073565).

Southern and Northern hybridization

Genomic DNA from *Hydra* polyps was isolated by tissue homogenization in 10 mM Tris (pH 8), addition of 0.5% sodium dodecyl sulfate (SDS), proteinase K treatment (100 µg/ml) for 2 h at 55°C, chloroform-phenol extraction, and ethanol precipitation. After a washing step in 75% ethanol, DNA was redissolved in 10 mM Tris (pH 8). Total RNA was isolated using TRIzol (Invitrogen). For *H. viridis*,

Fig. 1 *PPOD2*, a *PPOD1* homolog in *H. vulgaris* (Zürich), *H. vulgaris* (Basel), and *H. magnipapillata*. **a** Expression of *PPOD2* in steady state polyps. *PPOD2* in situ hybridization. **b** Complementary expression patterns of *PPOD1* and *PPOD2*. Double in situ hybridization. *Red precipitates* demonstrate the presence of *PPOD2* transcripts, *blue precipitates* demonstrate the presence of *PPOD1* transcripts. *Arrowheads* indicate borders of expression domains. **c** Tissue fractions used for RNA isolation in Northern blot and RT-PCR experiments (**d, e**, Figs. 2d and 5d). **d** *PPOD2* is alternatively spliced in *H. vulgaris* (Basel) and *H. magnipapillata*. Northern blot with total RNA of different *Hydra* species of the Vulgaris group. Hybridization with *H. vulgaris* (Basel) *PPOD1* and *PPOD2* probes. The *PPOD1* probe cross-hybridizes with *PPOD2* full-length transcripts under the conditions used. *Arrowhead* indicates *PPOD2* splice variant. **e** The *PPOD2* splice variants show no differential localization. RT-PCR with *H. vulgaris* (Basel) mRNA and *PPOD2*-specific primers. **f** Schematic comparison of common *PPOD* protein structure and short *PPOD2* splice variant. Note that the probe used for in situ hybridizations shown in **a** and **b** was common to both splice variants



a modified protocol was used (Habetha and Bosch 2005). For pulsed field gel electrophoresis, inert agarose-embedded DNA was digested. Restricted DNA was separated on 0.6% agarose gels or by pulsed field gel electrophoresis on 1% agarose gels with the PFGE CHEF II system from BIO-RAD (pulse 5–15 s, angle 120°, 18 h, 6 V/cm, 14°C). Total RNA was separated by formaldehyde agarose gel electrophoresis (Sambrook et al. 1989). 10 to 15 µg of genomic DNA and 15–30 µg of total RNA were taken per lane. RNA amounts were equilibrated by methylene blue staining of the membranes (data not shown). Southern and Northern transfers were carried out following standard procedures (Sambrook et al. 1989). Nucleic acids were transferred to Hybond N⁺ nylon membranes (Amersham Biosciences). Hybridizations

were carried out overnight in Church buffer at 55°C followed by washes in 0.2× standard saline citrate (SSC)/0.1% SDS at room temperature, 42 and 60°C for 2× 30 min, depending on the signal/background ratio. Autoradiographies were performed using phosphorimaging plates and the Phosphorimager FLA-5000 (FUJI). DNA probes were radio-labeled with α-[³²P]-dCTP using the Megaprime DNA Labeling System (Amersham Biosciences). If not specified in the text, the *PPOD* gene family probe was used in Northern and Southern hybridizations, representing a mixture of PCR fragments covering exons 2 of *H. vulgaris* (Basel) *PPOD1*, *PPOD2*, and *PPOD2*-like. The *PPOD* gene-specific probes covered the following *H. vulgaris* (Basel) *PPOD* gene 5'-flanking sequences in relation to the translational start (+1):

nucleotides -913 to -528 for *PPOD1*, -1164 to -909 for *PPOD2*, and -913 to -387 for *PPOD?* (see Fig. 3a). The *H. oligactis* probes used for the hybridizations shown in Fig. 5a corresponded to *PPOD1* (*H.oli.*) genomic nucleotides -750 to -592 and the cDNA nucleotides +72 to +633.

In situ hybridization

Dig-/Biotin-labeled riboprobes were synthesized using RNA Labeling Kits from Roche and combined with anti-Dig-AP/anti-Biotin-POD antibodies (Roche). In situ hybridizations were performed as described before (Martinez et al. 1997). The hybridization temperature was 64°C. Performing double in situ hybridizations, Dig-labeled probes were added 12 h after the addition of Biotin-labeled probes. Using Biotin-labeled probes, endogenous peroxidases were inhibited by incubation in 3% hydrogen peroxide in 2× SSC/0.1% (w/v) CHAPS for 30 min after the removal of RNA probes. Efficiency of peroxidase inhibition was monitored by peroxidase staining of polyps that had not been incubated with the antibody (Fig. 1b, control). Alkaline phosphatase staining was carried out with Nitro Blue Tetrazolium/5-bromo-4-chloro-3'-indolyl-phosphate *p*-toluidine salt (NBT/BCIP) in NTMT buffer (1:100). Peroxidase staining was carried out with 0.3 mg/ml diaminobenzidine (DAB) in 100 mM Tris (pH 7.3). After 10 min of preincubation, the peroxidase staining was started by the addition of 0.01% (w/v) hydrogen peroxide. The peroxidase staining reaction was stopped by several washes in phosphate-buffered saline/Tween 20 (PBT)/1 mM sodiumazide. Animals were dehydrated prior to euparal embedding. The riboprobes corresponded to different parts of *PPOD* gene cDNA sequences from various species [nucleotide positions in relation to the translational start (+1)]: *H. vulgaris* (Basel) *PPOD1* and *PPOD2* (see Hoffmeister-Ullerich et al. 2002); for *H. magnipapillata* *PPOD1* (-80 to +222) and *PPOD2* [= *H. vulgaris* (Basel) probe]; for *H. vulgaris* (AEP) (+40 to +535); for *H. oligactis* *PPOD1* (+26 to +634). The latter has been also used for *H. robusta*.

Peroxidase assay

The peroxidase assay was carried out as described before (Hoffmeister and Schaller 1985). Stained animals were dehydrated and embedded in euparal.

Sequence analysis

Expressed sequence tag (EST) analysis was performed using the sputnik platform (<http://sputnik.btk.fi>) and the *H. vulgaris* (Basel) *PPOD1* cDNA sequence as query. The Seqtools program was used for sequence analysis. Conservation profiles for 5'-flanking *PPOD* gene sequences were generated with the ConSite platform (<http://phylofoot.org/>; Lenhard et al. 2003; Sandelin et al. 2004a,b).

Results

PPOD1 and *PPOD2* show complementary expression patterns in *H. vulgaris* (Basel) and *H. magnipapillata*

PPOD1 and *PPOD2* have been isolated and characterized in *H. vulgaris* strain Zürich (S. Hoffmeister-Ullerich, personal communication). For our analysis, we recloned both genes from *H. vulgaris* (Basel). We observed no (*PPOD1*) and one (*PPOD2*) amino acid exchange when compared to the *H. vulgaris* (Zürich) sequences (data not shown). Figure 1 shows the expression of *PPOD2* for *H. vulgaris* (Basel). *PPOD2* is expressed in the epithelial cells of the ectoderm of the whole body column and the tentacle bases. No transcripts could be detected in the hypostome, the tentacles, and the basal disk. *PPOD2* transcripts are found down to the junction of the peduncle and the basal disc (Fig. 1a, lower left, arrowheads). Thus, consistent with the results of the previous study (Hoffmeister-Ullerich et al. 2002), *PPOD2* and *PPOD1* show strikingly complementary expression patterns in the foot region. Double in situ hybridization shows that the expression domains of both genes are separated by the previously described differentiation border (e.g. Dübel et al. 1987) between the peduncle and the basal disc (Fig. 1b, arrowheads).

The analysis of the *H. magnipapillata* EST database (<http://sputnik.btk.fi>) revealed four major *PPOD* gene clusters. Consensus sequences were generated; full-length cDNAs including parts of the untranslated regions were recloned and named *PPOD1* (*H.mag.*), *PPOD2* (*H.mag.*), *PPOD2*-short (*H.mag.*), and *PPOD2*-like (*H.mag.*) due to their similarities to the known *PPOD* genes in *H. vulgaris* (Zürich) and *H. vulgaris* (Basel) (Supplementary Fig. S1). At the nucleotide level, *PPOD1* (*H.mag.*) and *PPOD2* (*H.mag.*) show 90 and 99% identity, respectively, with the *H. vulgaris* (Basel, Zürich) genes (Supplementary Fig. S1b). The expression patterns in *H. magnipapillata*, as monitored by in situ hybridization, were identical to the ones determined for the *H. vulgaris* (Basel) genes (data not shown). Thus, they are considered to be homologs. *PPOD2*-short is most likely a *PPOD2* splice variant, as it shows sequence identities to *PPOD2* also in 3'- and 5'-untranslated regions (Supplementary Fig. S2). The presence of a shorter *PPOD2* splice variant in some species of the *Vulgaris* group, namely, *H. vulgaris* (Basel) and *H. magnipapillata*, could be confirmed by sequencing (Supplementary Fig. S2), Northern blotting (Fig. 1d, arrowheads), and RT-PCR (Fig. 1e). In addition, RT-PCR suggests that the splice variants show no differential expression patterns. The alternative splicing of *PPOD2* leads to a truncated protein (Fig. 1f) lacking the first three of six deduced modules common for *PPOD1* and *PPOD2* from *H. vulgaris* (Zürich) (Hoffmeister-Ullerich et al. 2002) and all other *PPOD* genes cloned from different species in the course of this study (Supplementary Fig. S3). *PPOD2*-like (*H.mag.*) shows 85% identity to *PPOD2* (data not shown). The expression pattern of this additional *PPOD* gene remains to be shown. In sum, there are at least three *PPOD* genes expressed in *H. vulgaris* (Basel) and *H. mag-*

nipapillata. *PPOD1* and *PPOD2* seem to be closely related genes with complementary expression patterns in the foot region of *H. vulgaris* (Zürich), *H. vulgaris* (Basel), and *H. magnipapillata*. Moreover, the foot-specific differentiation of ectodermal epithelial cells in steady state polyps appears to coincide with an inhibition of *PPOD2* expression and the transcriptional activation of *PPOD1* expression.

PPOD1 and *PPOD2* genes are tightly regulated during foot differentiation in *H. vulgaris* (Basel)

To investigate the dynamics of this complementary expression in various developmental situations, we used *H. vulgaris* (Basel) and performed in situ hybridizations with *PPOD1* and *PPOD2* probes and a peroxidase assay (Fig. 2) to visualize the activity of the basal disc peroxidase, which most likely represents the active *PPOD1* gene product (Hoffmeister-Ullerich et al. 2002). During budding, both *PPOD1* transcripts and the peroxidase activity can be detected only at very late stages close to detachment, as has been reported before (Fig. 2a, closed arrowheads; Hoffmeister-Ullerich et al. 2002). From very early stages on, *PPOD2* transcripts are absent from the future head tissue (stages 3–5), developing head structures like tentacle anlagen (stages 6–8), and the hypostome (stages 9 and 10) of the developing bud (Fig. 2a, open arrowheads). This down-regulation correlates with the early onset of expression of patterning

genes thought to play a role in the *Hydra* head organizer (for recent review, see Broun and Bode 2002) and suggests that the restriction of *PPOD2* to the body column of *Hydra* (Fig. 1a) is based on negative signals from the head. In the foot region of the developing bud, *PPOD2* seems to be down-regulated slightly before *PPOD1* transcripts can be detected (Fig. 2a). In late buds, the adult *PPOD2* pattern is established.

Foot regeneration includes wound closure and morpholactic regeneration by differentiation of gastric epithelial cells into basal disc cells (for recent review, see Galliot and Schmid 2002; Holstein et al. 2003). Consistent with previous reports (Hoffmeister and Schaller 1985; Hoffmeister-Ullerich et al. 2002), these events are accompanied by an early onset of *PPOD1* expression as well as the peroxidase activity (Fig. 2b). After 36 h, the original *PPOD1* transcript level (see Fig. 1b) is restored. After wound closure, *PPOD2* can be detected in all ectodermal epithelial cells of the regenerating stump (not shown). After 24 h, single cells devoid of *PPOD2* transcripts become visible (Fig. 2b, open arrowhead). The adult pattern is reestablished after 36 h.

Long-term incubation of certain *H. vulgaris* strains with low concentrations of lithium chloride leads to ectopic formation of foot structures (Hassel and Berking 1990; Hassel et al. 1993). As shown in Fig. 2c, numerous ectopic basal discs formed along the body column of *H. vulgaris* (Basel) following incubation in 0.5 mM LiCl. Appearance of ectopic feet strongly affects *PPOD1* and *PPOD2* ex-

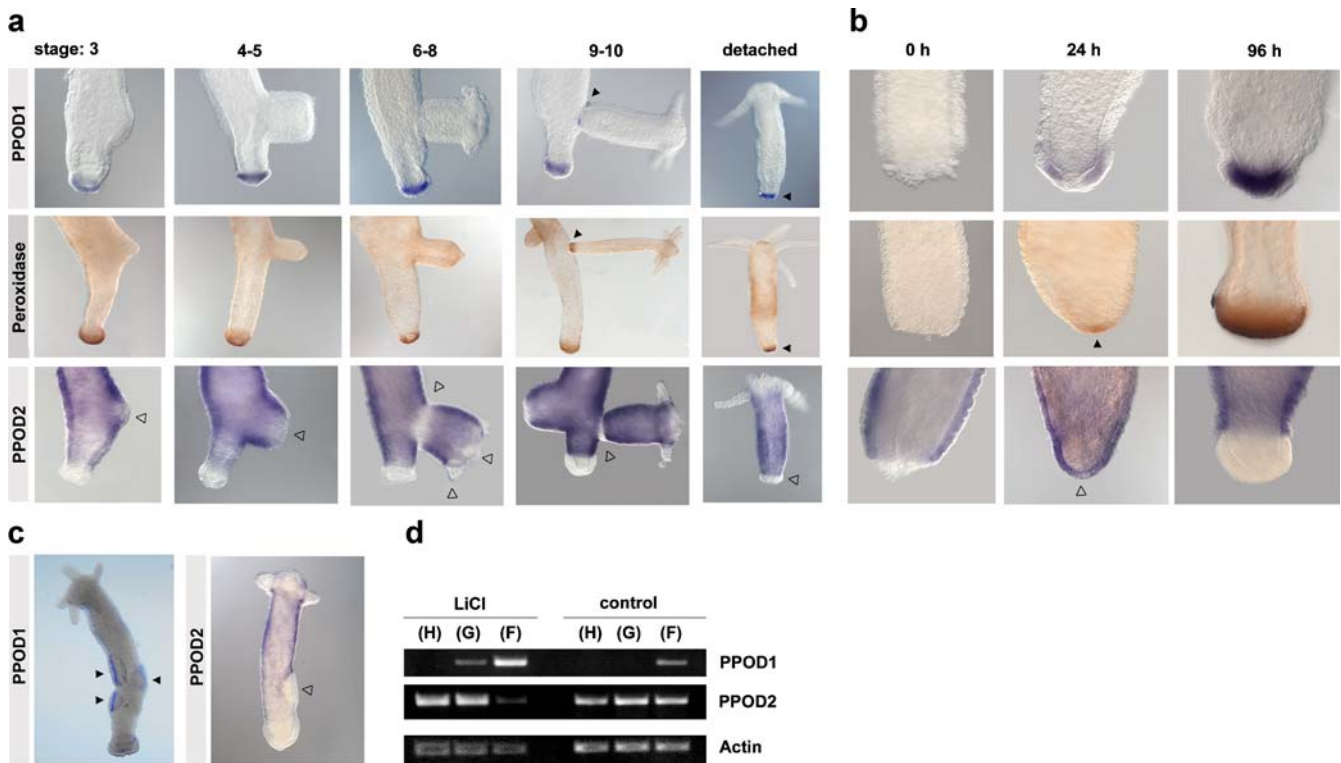


Fig. 2 The complementary expression of *PPOD1* and *PPOD2* is maintained during various foot differentiation events in *H. vulgaris* (Basel). **a** Budding. Stages according to Otto and Campbell (1977). In situ hybridization with *PPOD1* and *PPOD2* probes; middle panels show additional peroxidase assay. **b** Foot regeneration

(amputation in midpeduncle region). Regenerating stumps after 0, 24, and 96 h. **c, d** Ectopic foot formation upon LiCl treatment (0.5 mM LiCl, 14 days). **d** RT-PCR with *PPOD1*- and *PPOD2*-specific primers [RNA from head (H), gastric region (G), and foot tissue (F); see Fig. 1c]

pression (Fig. 2c, compare to Fig. 1a,b). The onset of *PPOD1* expression in the peduncle and gastric region preceded the visible ectopic structures (data not shown) and was also monitored by RT-PCR (Fig. 2d). Moreover, the formation of ectopic basal discs led to a loss of *PPOD2* transcript in the foot region of LiCl-treated polyps (Fig. 2d). Taken together, these data show that the complementary expression patterns of both *PPOD1* and *PPOD2* are maintained during foot differentiation events in different developmental contexts.

There are several *PPOD* genes in the *H. vulgaris* (Basel) genome

The identification of two closely related *PPOD* genes in *H. vulgaris* (Zürich) (Hoffmeister-Ullerich et al. 2002) pointed to the existence of a gene family. To examine that in more detail, we performed Southern blot analysis to characterize *PPOD* genes at the genomic level in *H. vulgaris* (Basel) using both standard agarose gel electrophoresis (Fig. 3b) and pulsed field gel electrophoresis (Fig. 3c).

Cloning and sequencing of genomic *PPOD* fragments from *H. vulgaris* (Basel) and *H. magnipapillata* revealed the common intron/exon structure depicted in Fig. 3a (see also Supplementary Figs. S1, S2, S3). Filters were subsequently hybridized with a gene family probe (Fig. 3a, gray bar) and probes specific for single *PPOD* genes (Fig. 3a, black, streaked, and white bars). Several signals were obtained with the gene family probe (Fig. 3b, gray bar). As expected, hybridization of the same filter with gene-specific probes yielded subsets of the initially observed signals. Numerous signals for single *PPOD* genes (Fig. 3b, black, streaked bars) suggest the presence of multiple copies for some of the genes. Restriction sites within the introns (see Fig. 3a) may account for gene-specific signals with no counterpart in the initial gene family hybridization. Similar results like the ones shown in Fig. 3b were obtained with other restriction enzymes (data not shown). The smallest fragment giving a signal both for the gene family and the *PPOD1* probe and thus representing a fragment with a complete gene (compare Fig. 3a) had a size of 7 kbp and was observed with *Bgl*II digestion (data not shown). Thus, with respect to the position of the probes (Fig. 3a), the *PPOD1* intron spans approximately 5 kbp. Supporting the EST analysis, several *PPOD* genes including *PPOD1* and *PPOD2* could be detected at the genomic level. Our data also indicate that *PPOD1* and *PPOD2* exist in more than one copy. Moreover, the presence of high molecular weight restriction fragments with signals for more than one *PPOD* gene (Fig. 3b,c, arrowheads), which were observed also using alternative restriction enzymes (*Bgl*III, *Xba*I, *Eco*105I, *Bsu*15I; data not shown), is consistent with the view that *PPOD* genes are clustered in the *H. vulgaris* (Basel) genome. Direct evidence for a clustering of *PPOD* genes might come from the assembly of the *H. magnipapillata* genome in the near future.

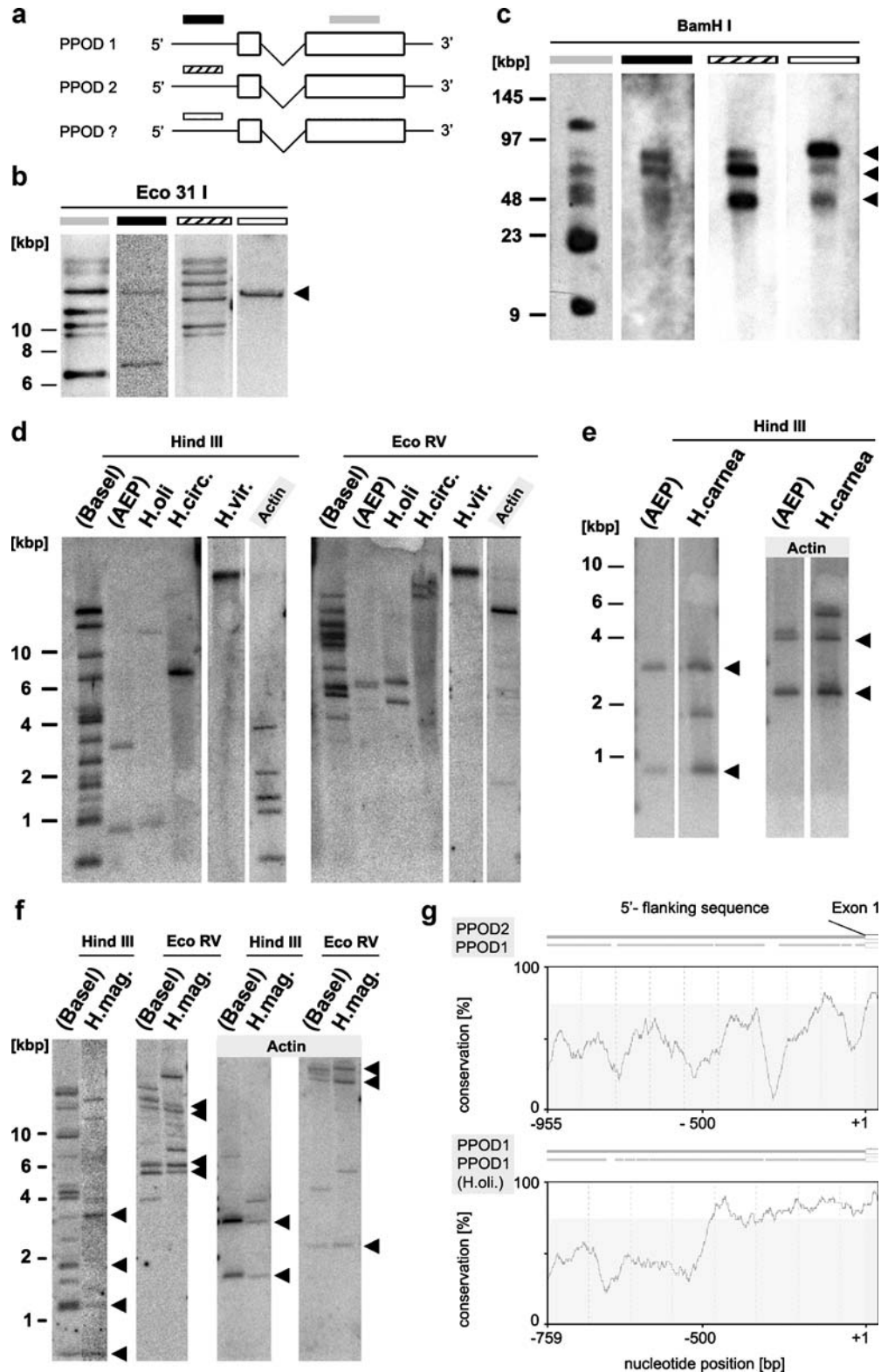
Genomic complexity of the *PPOD* gene family in different *Hydra* species

The basal-disc-specific peroxidase activity, thought to be due to the gene expression of *PPOD1* (Hoffmeister-Ullerich et al. 2002), is present in all *Hydra* species (Hoffmeister and Schaller 1985; Galliot 1997; personal observation). To examine whether a common *PPOD1*-like gene is present in these species, we analyzed the *PPOD* gene family at the genomic level in *Hydra* species representing all four taxonomic groups (Fig. 3d–f). As observed before (Fig. 3b,c), several signals were obtained for *H. vulgaris* (Basel), while another species of the Vulgaris group, *H. vulgaris* (AEP), and putatively more basal species such as *H. oligactis*, *H. circumcincta*, and *H. viridis* yielded only one to two bands in different restrictions (Fig. 3d). For *H. viridis*, a common high molecular weight signal was detected. This signal was also detected using restrictions with other enzymes (*Xba*I, *Clal*, *Eco*RI; data not shown). Since ethidium bromide staining of the agarose gel suggested complete DNA restrictions (data not shown) and since control hybridizations with actin (Fig. 3d) and GAPDH (data not shown) probes showed clear low molecular weight signals, the high molecular weight *PPOD* signals for *H. viridis* appear to be specific and may indicate that the *PPOD* gene(s) in *H. viridis* is (are) located in a genomic region not accessible for common restriction enzymes.

The differential complexity of *PPOD* genes among closely related species in the Vulgaris group was surprising and led us to include two more species, *H. magnipapillata* and *H. carnea*, in our analysis. Phylogenetic analysis of ribosomal sequences (D. Martinez, personal communication) suggests a close relationship of *H. magnipapillata* and *H. vulgaris* (Basel), while *H. carnea* groups together with *H. vulgaris* (AEP) on a separate branch of the phylogenetic tree (see Fig. 6). Southern blot experiments with the *PPOD* gene family probe as well as with an actin probe support this phylogenetic relationships (Fig. 3e,f). *H. magnipapillata* and *H. vulgaris* (Basel) both show numerous *PPOD* signals, while *H. carnea* and *H. vulgaris* (AEP) show only two to three signals. Moreover, *H. carnea* and *H. vulgaris* (AEP) (Fig. 3e) as well as *H. magnipapillata* and *H. vulgaris* (Basel) (Fig. 3f) show several common restriction fragments (arrowheads), pointing to closely related genomic sequences with common restriction sites. These findings suggest multiple gene duplication events within the Vulgaris group, a cluster of species with many common taxonomically relevant features.

The strikingly complementary expression patterns of *PPOD1* and *PPOD2* (see Fig. 1) suggest major sequence differences in the 5'-regulatory flanking sequences of both genes. To examine that, we used a global alignment of *PPOD1* and *PPOD2* 5'-flanking regions from *H. vulgaris* (Basel) as well as the *PPOD1* 5'-flanking regions from *H. vulgaris* (Basel) and *H. oligactis* to generate the conservation profiles shown in Fig. 3g. In contrast to the low degree of sequence identity among the 5'-flanking sequences of *PPOD1* and *PPOD2* in *H. vulgaris* (Basel), the 5'-flanking sequence of *PPOD1* shows a high degree of interspecific

Fig. 3 *PPOD* genes at the genomic level within the genus *Hydra*. **a** Schematic representation of *PPOD* gene intron/exon structure as determined for *H. vulgaris* (Basel) and *H. magnipapillata*. Bars indicate hybridization probes used in Southern blot analyses. Gene family probe (gray) corresponds to conserved exon 2; gene-specific probes (black, streaked, and white) correspond to variable 5'-flanking regions. *PPOD?*, unknown *PPOD* gene. **b, c** Single *PPOD* genes are present in multiple copies. Southern blots subsequently hybridized with *PPOD* gene family and gene-specific probes provide hints for genomic clusters of *PPOD* genes in the *H. vulgaris* (Basel) genome. Arrowheads indicate *PPOD* fragments with signals for more than one *PPOD* gene. **d-f** Southern blots hybridized with *PPOD* gene family and control probe (*H. viridis* Actin), respectively. **d** Complexity of *PPOD* gene family in different *Hydra* species. Control hybridization shown for the *H. viridis* lane (see text for details). **e, f** Variable complexity of *PPOD* gene family within the Vulgaris group. Arrowheads indicate common restriction fragments. **e** Close relationship of the North American Vulgaris species *H. vulgaris* (AEP) and *H. carnea*. **f** Close relationship of the European *H. vulgaris* (Basel) and the Japanese *H. magnipapillata*. **g** Differential expression of *PPOD1* and *PPOD2* is reflected by diverse 5'-regulatory regions. Note the high degree of conservation among the first 450 bp of orthologous *PPOD1* 5'-flanking regions in *H. vulgaris* (Basel) and *H. oligactis* (lower panel). Shaded in gray, 75% conservation (graphics modified from <http://phylofoot.org/>). Species abbreviations: Basel=*H. vulgaris* (Basel), AEP=*H. vulgaris* (AEP), *H.oli.*=*H. oligactis*, *H. circ.*=*H. circumcincta*, *H.vir.*=*H. viridis*, *H.mag.*=*H. magnipapillata*



conservation within the first 450 bp. These intra- and inter-specific sequence comparisons suggest (1) that the differential expression of *PPOD1* and *PPOD2* (Figs. 1b and 2) is genomically hardwired in differential regulatory sequences and (2) that crucial elements for the basal-disc-specific ex-

pression of *PPOD1* in *H. vulgaris* and *H. oligactis* (Figs. 1b and 5b) might be found in the conserved region.

In sum, genomic as well as cDNA sequences of *PPOD* genes from various *Hydra* species revealed many variations within even closely related species. The data support a

recently proposed phylogenetic tree for the genus *Hydra* and provide first insight into the molecular basis of differential *PPOD* gene expression in *H. vulgaris* (Basel).

PPOD genes in sexual *H. vulgaris* (AEP) animals

To investigate the expression of *PPOD* genes in Northern American *Vulgaris* species and putatively more basal groups of the genus *Hydra*, we isolated *PPOD* genes from *H. vulgaris* (AEP) and *H. oligactis* using RT-PCR with primers against conserved parts of the known *H. vulgaris* (Basel), *H. vulgaris* (Zürich), and *H. magnipapillata* genes. Full-length cDNA sequences were obtained by 5'- and 3'-RACEs (see “Materials and methods”).

All *PPOD* fragments generated by RT-PCR with *H. vulgaris* (AEP) mRNA could be assigned to a single transcript, which is consistent with only two signals in the Southern blot (Fig. 3d,e). The *H. vulgaris* (AEP) cDNA sequence shows highest sequence identity to *H. vulgaris* (Basel) *PPOD1* and, thus, has been assigned *PPOD1* (AEP). In addition to a strong expression in the basal disc and in contrast to observations in *H. vulgaris* (Basel), *H. magnipapillata* (Figs. 1b and 2a–c), and *H. oligactis* (Fig. 5b), *PPOD1* (AEP) shows weak but significant expression in the gastric region (Fig. 4a). This is further supported by the Northern blot shown in Fig. 1d. It seems unlikely that the *PPOD1* (AEP) pattern is a result of cross-hybridization with another, yet unknown *PPOD* gene since the high stringent in situ hybridization conditions applied here allow to discriminate the expression patterns of different *PPOD* genes with even high degrees of sequence identity in *H. vulgaris* (Basel) (see Fig. 1a,b). The gastric expression of *PPOD1* (AEP) was also observed in *H. carnea* (Fig. 4b), a closely related species according to ribosomal sequence analysis and the data shown in Fig. 3e. As observed for *PPOD2* in *H. vulgaris* (Basel)

(Fig. 1a,b), no *PPOD1* (AEP) transcripts were found in the hypostome and the tentacles. The expression pattern of *PPOD1* (AEP), thus, resembles an “overlay” of the *PPOD1* and *PPOD2* expression domains in *H. vulgaris* (Basel).

The assay used to visualize the basal-disc-specific peroxidase (Hoffmeister and Schaller 1985; Hoffmeister-Ullerich et al. 2002; Fig. 2a,b) reveals additional peroxidase activities along the body column (Hoffmeister-Ullerich et al. 2002). In female polyps, a distinct activity resides in the engulfed nurse cells of developing oocytes and embryos (Technau et al. 2003; Fig. 4c). In male polyps, we observed an activity restricted to the tips of the testis (Fig. 4c). In situ hybridization with *PPOD1* (AEP) (Fig. 4c) and RT-PCR with mRNA from budding and female polyps as well as different embryo stages (Fig. 4d) show that these peroxidase activities do not correlate with elevated levels of *PPOD* gene expression in *H. vulgaris* (AEP) pointing to the activity of yet unknown enzymes.

PPOD genes in *H. oligactis* and other putatively basal *Hydra* species

As observed for *H. vulgaris* (AEP), all *PPOD* fragments generated by RT-PCR from *H. oligactis* mRNA could be assigned to a single transcript, *PPOD1* (*H.oli.*), with highest sequence similarity to *PPOD1* from *H. vulgaris* (Basel). This is consistent with the detection of only two signals in Southern blot experiments using DNA from *H. oligactis* (Figs. 3d and 5a) and *H. robusta*, another species from the *Oligactis* group (data not shown). Subsequently hybridizing genomic DNA from *H. oligactis* with a probe corresponding to exon 2 and a probe corresponding to the 5'-flanking region of the same gene (Fig. 5a) yielded identical bands for some restrictions (Fig. 5a). Since different *PPOD* genes showed strong variations in their 5'-flanking regions in *H.*

Fig. 4 Expression of *PPOD* genes in two North American *Vulgaris* species. **a–c** In situ hybridizations with *PPOD1* (AEP)—probe; in **c**, an additional peroxidase assay is shown. **a** Asexual *H. vulgaris* (AEP) polyp. **b** Asexual *H. carnea* polyp. **c, d** Enhanced peroxidase activities during oogenesis/embryogenesis and spermatogenesis do not correspond to enhanced levels of *PPOD1* (AEP) expression. **c** Sexual *H. vulgaris* (AEP). Arrowheads point to developing gonads. **d** RT-PCR with RNA from budding polyps, female (eggfleck bearing) polyps, and different embryo stages. *PPOD1* (AEP)-specific primers were used

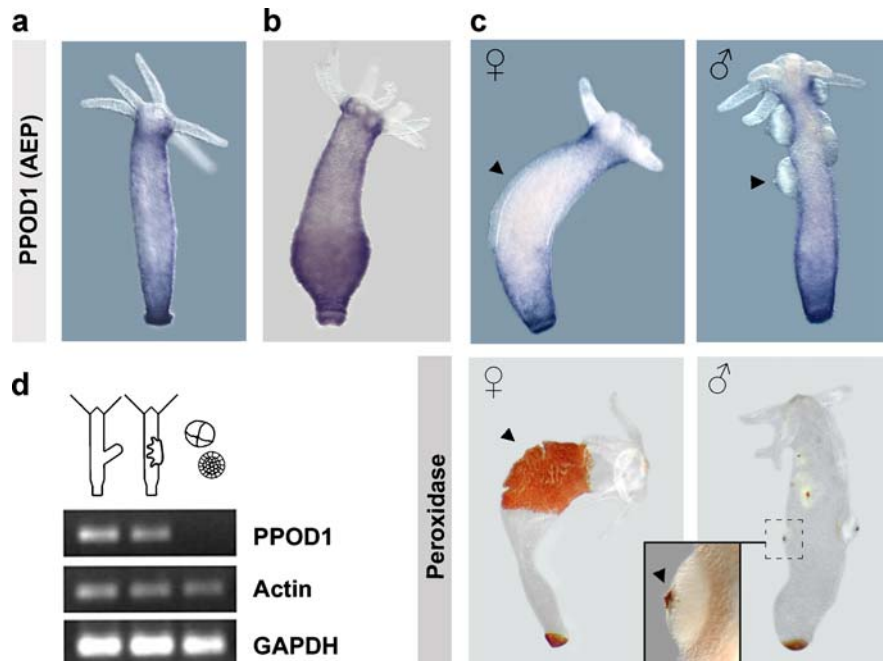
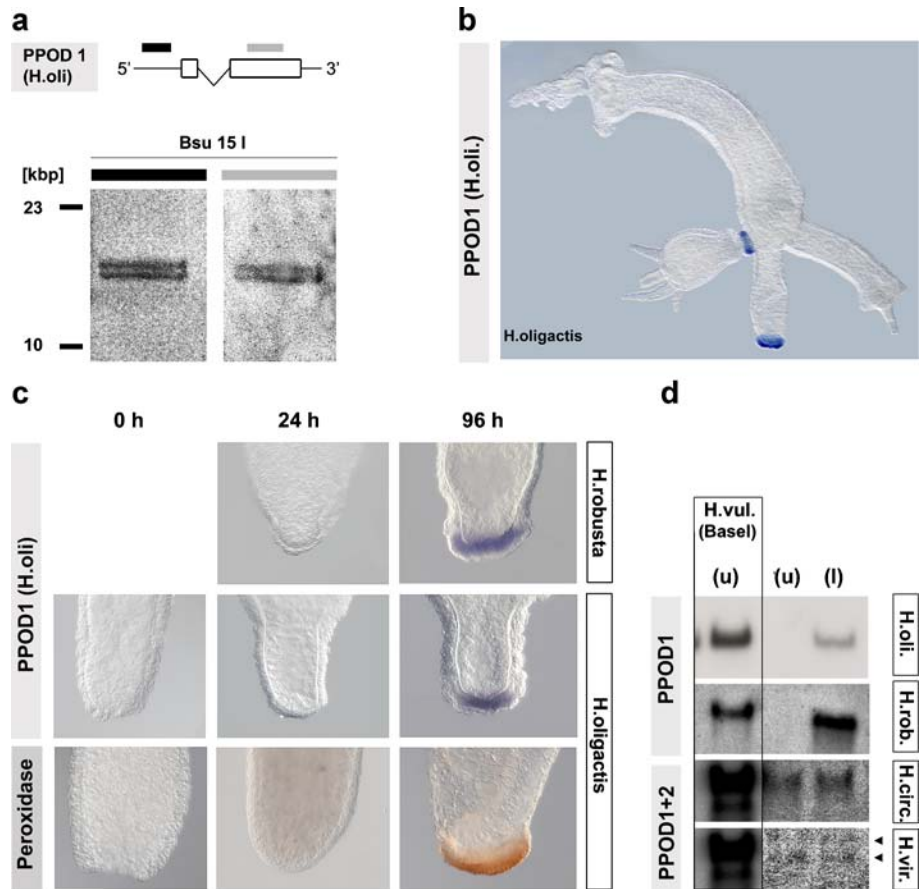


Fig. 5 *PPOD1* in basal *Hydra* species. **a** *H. oligactis* contains one *PPOD* gene in two copies. Southern blot with *PPOD1* (*H. oli.*) probes corresponding to 5'-flanking region and exon 2. **b** Basal-disc-specific expression of *H. oligactis PPOD1*. In situ hybridization with *PPOD1* (*H. oli.*) probe. **c** Foot regeneration deficiency of Oligactis group species monitored by *PPOD1* (*H. oli.*) in situ hybridization and peroxidase assay. Amputation in midpeduncle region. Compare to Fig. 2b. **d** The expression of *PPOD* genes in basal *Hydra* species. Northern blot with *H. vulgaris* (Basel) *PPOD1* and *PPOD2* probes and total RNA from different *Hydra* species. Arrowheads indicate weak signals in *H. viridis*. Species abbreviations: *H. oli.* = *H. oligactis*, *H. rob.* = *H. robusta*, *H. circ.* = *H. circumcincta*, *H. vir.* = *H. viridis*, *H. vul.* = *H. vulgaris*



vulgaris (Basel) (Fig. 3a,g, see above), the observations shown in Fig. 5a indicate that the two bands observed in Southern blots with *H. oligactis* DNA account for two copies of the same gene. Similar to its homologs in *H. vulgaris* (Basel), *H. vulgaris* (Zürich), and *H. magnipapillata* (Figs. 1b and 2a,b), the expression of *PPOD1* (*H. oli.*) is restricted to the basal disc in *H. oligactis* and *H. robusta* (Fig. 5b,d). *PPOD1* shows similar expression dynamics in both *H. vulgaris* (Basel) and *H. oligactis* during budding and regeneration (Fig. 5b,c, compare to Fig. 2a,b). Previously, a delay in foot regeneration has been described for *H. oligactis* when compared to species of the Vulgaris group (Hoffmeister 1991). When analyzing both the expression of *PPOD1* (*H. oli.*) and the basal disc peroxidase during foot regeneration in both *H. oligactis* and *H. robusta* (Fig. 5c) by in situ hybridization and peroxidase assay, respectively, in contrast to *H. vulgaris* (Basel) (see Fig. 2b), no detectable amounts of *PPOD1* (*H. oli.*) mRNA and peroxidase activity were observed after 24 h and even 36 h (not shown). In sum, there is only one *PPOD* gene present in *H. oligactis* and *H. robusta*. Functional basal disc formation appears to be correlated with the restoration of normal *PPOD1* (*H. oli.*) expression pattern after 96 h of regeneration.

The presence of a peroxidase activity in the basal discs of *H. circumcincta* and *H. viridis*, most likely a result of *PPOD1* expression in *H. vulgaris* (Zürich) (Hoffmeister-Ullerich et al. 2002), as well as the Southern blot experiments (Fig. 3d) suggested the presence of *PPOD* genes

also in *H. circumcincta* and *H. viridis*. Northern blot experiments with a heterologous *PPOD* probe from *H. vulgaris* (Basel) showed weak signals for *H. circumcincta* and even weaker signals for *H. viridis* in the expected size range in both upper and lower half of the animal [Fig. 5d, blot pictures for *H. circumcincta* and *H. viridis* were increasingly contrasted, note the strong signals using *H. vulgaris* (Basel) RNA for comparison]. Since *PPOD* genes seem to be expressed in the gastric region of both *H. circumcincta* and *H. viridis* and since these species are thought to have diverged early during *Hydra* phylogeny, this might reflect the ancestral situation. Neither homolog hunting by RT-PCR nor the screening of a cDNA library containing *H. viridis* cDNA, however, resulted in the identification of *PPOD* homologs in *H. circumcincta* and *H. viridis* (data not shown). Consistent with the weak signals in the Northern blot (Fig. 5d), this may be due to a large degree of sequence divergence in these species.

Discussion

PPOD genes and *Hydra* peroxidase activities

PPOD1 has been identified by Sabine Hoffmeister-Ullerich et al. as the gene most likely corresponding to the basal disc peroxidase activity in *H. vulgaris* (Zürich), which was originally introduced as a biochemical marker for differen-

deficiency of *H. oligactis* (Hoffmeister 1991) and propose it as a common feature of the Oligactis group.

PPOD2 is a suitable marker for peduncle tissue (Fig. 1a,b) in *H. vulgaris* (Basel), *H. vulgaris* (Zürich), and *H. magnipapillata*. The strikingly differential regulation in comparison to *PPOD1* (Figs. 1 and 2) is particularly interesting as both genes seem to be located in close vicinity to each other in a cluster of *PPOD* genes within the *H. vulgaris* (Basel) genome (Fig. 3a–c). Although it remains to be shown whether both genes originated from a recent gene duplication, it is interesting to note that complementary expression of closely related genes originating apparently from a single ancestral gene has been also observed for two other *Hydra* genes, *Hym-301* (K. Khalturin, personal communication), a peptide with a proposed role in tentacle formation (Takahashi et al. 2005), and *Hym 176/Hym 357*-related genes (Fujisawa, personal communication). A comparative analysis of the regulatory regions of *PPOD1* and *PPOD2* in *H. vulgaris* (Basel), therefore, promises to enhance our understanding of basal-disc- and gastric-region-specific gene expression.

PPOD genes, genomic plasticity, and phylogeny of *Hydra*

About 25 *Hydra* species have been well described (Campbell 1987); taxonomy groups them into four species clusters, namely, the Viridis, Circumcincta, Oligactis, and Vulgaris groups (Campbell 1987; 1989; Holstein and Campbell 1990). Histocompatibility studies (reviewed in Holstein 1995; Kuznetsov et al. 2002) as well as studies on chromosome morphology and genome size measurements (Zacharias et al. 2004 and references within) support this concept and point to a close relationship of the Oligactis and Vulgaris groups and to an early divergence of the Viridis and Circumcincta groups. Studies using 18S rRNA (Collins 2000) as well as the internal transcribed spacer (ITS 1, ITS 2) of *Hydra* nuclear ribosomal RNAs (D. Martinez, personal communication) are consistent with this view and support the phylogenetic tree depicted in Fig. 6.

In *H. vulgaris* (Basel) and *H. magnipapillata*, our studies revealed at least three *PPOD* genes, partly in multiple copies (Figs. 3b,c and 6), while apparently only one *PPOD1*-like gene is present in the North American Vulgaris group species *H. vulgaris* (AEP) and *H. carnea*. *H. vulgaris* (AEP) was generated under laboratory conditions (Martin et al. 1997), while *H. carnea* is a well-described species (Hyman 1931; Campbell 1987). Beside *H. vulgaris* (Basel) and *H. magnipapillata*, *H. vulgaris* (AEP) is a main model organism in the *Hydra* field. Concerning taxonomically relevant morphological features, it is virtually indistinguishable from *H. vulgaris* (Basel) and *H. magnipapillata* (B. Anokhin, personal communication). A recent study on genome sizes in a number of *Hydra* species revealed a strong correlation between chromosome and genome sizes (Zacharias et al. 2004). *H. vulgaris* (AEP) exhibits (i) identical chromosome numbers and (ii) chromosome as well as genome sizes comparable to *H. vulgaris* (Basel) and *H. magnipapillata*

(Zacharias et al. 2004; B. Anokhin, personal communication). Thus, the increase in complexity within the *PPOD* gene family apparently resulted from “local” gene duplication events rather than genome duplications. Compared with other species of the Vulgaris group, *H. vulgaris* (AEP) remarkably shows reduced signal numbers in Southern blots also for other genes (G. Ghenikhovich, personal communication). These findings potentially reflect a higher degree of gene redundancy in *H. vulgaris* (Basel) and *H. magnipapillata*, which might hamper functional approaches in these species.

Similar observations on the *PPOD* gene number were made for *H. oligactis* and *H. robusta*, two species of the Oligactis group. As the basal-disc-specific peroxidase, most likely encoded by *PPOD1*, is present in all *Hydra* species monitored (Hoffmeister and Schaller 1985; Galliot 1997; personal observation) and since the low number of one or two signals on the Southern blots for *H. circumcincta* and *H. viridis* (Fig. 3d) corresponds to two signals in the *H. oligactis* blot (Fig. 3d), which could be assigned to a single *PPOD* gene (Fig. 5a), we conclude that there is also only one, most likely *PPOD1*-like, *PPOD* gene in these putatively basal species (Fig. 6).

The drastic variations in the structure of the *PPOD* gene family (Fig. 3) as well as the expression patterns of *PPOD1* within closely related species of the Vulgaris group (Figs. 1 and 2, compare to Fig. 4) support the recently suggested (D. Martinez, personal communication) close relationship of the North American species *H. vulgaris* (AEP) and *H. carnea* in comparison to the European Vulgaris strains Zürich and Basel and the Japanese *H. magnipapillata* (Fig. 6). The apparently strongly differing *PPOD* gene sequences in *H. circumcincta* and *H. viridis* are consistent with the small genome size, most probably a basal feature, in *H. viridis* (Zacharias et al. 2004; Fig. 6). They support the view of an early divergence of these species during *Hydra* phylogeny.

Taken together, our data on *PPOD* genes in various *Hydra* species support the recently proposed phylogeny for the genus *Hydra*. They suggest autonomous gene duplications in the course of *Hydra* genome evolution within the Vulgaris group and point to an unexpected genomic plasticity within closely related species of the genus *Hydra*.

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