Hym-301, a novel peptide, regulates the number of tentacles formed in hydra

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Summary

Hym-301 is a peptide that was discovered as part of a project aimed at isolating novel peptides from hydra. We have isolated and characterized the gene Hym-301, which encodes this peptide. In an adult, the gene is expressed in the ectoderm of the tentacle zone and hypostome, but not in the tentacles. It is also expressed in the developing head during bud formation and head regeneration. Treatment of regenerating heads with the peptide resulted in an increase in the number of tentacles formed, while treatment with Hym-301 dsRNA resulted in a reduction of tentacles formed as the head developed during bud formation or head regeneration. The expression patterns plus these manipulations indicate the gene has a role in tentacle formation. Furthermore, treatment of epithelial animals indicates the gene directly affects the epithelial cells that form the tentacles. Raising the head activation gradient, a morphogenetic gradient that controls axial patterning in hydra, throughout the body column results in extending the range of Hym-301 expression down the body column. This indicates the range of expression of the gene appears to be controlled by this gradient. Thus, Hym-301 is involved in axial patterning in hydra, and specifically in the regulation of the number of tentacles formed.

Key words: Hydra, Hym-301, Peptide, Tentacle formation

Introduction

An issue of interest in the evolution of developmental processes among metazoans concerns the extent of the conservation of molecular pathways and their roles in development. For example, the signalling pathways affecting most developmental processes in bilaterians are few in number. They include the Wnt, TGFβ, Hedgehog, Notch, receptor tyrosine kinase (RTK), nuclear hormone and JAK/STAT pathways (Pires-daSilva and Sommer, 2003). That these pathways arose fairly early in metazoan evolution is indicated by the presence of orthologues of genes of five of these seven pathways in hydra and other cnidarians. These include genes of the Wnt (Hobmayer, 1996; Hobmayer et al., 2000; Minobe et al., 2000), TGFβ (Hayward et al., 2002; Reinhardt et al., 2004), Hedgehog (K. Kaloulis, PhD Thesis, University of Geneva, 2000), RTK (Steele et al., 1996; Bridge et al., 2000; Steele, 2002) and nuclear hormone (Escriva et al., 1997) pathways. Several of these pathways are involved in axial patterning processes in hydra.

The animal consists of a single oral-aboral axis with radial symmetry. Along the axis are three regions: the head, body column and foot, or basal disk. Because of the tissue dynamics of an adult hydra, the processes governing axial patterning, morphogenesis, cell differentiation and cell division are constantly active (e.g. Bode, 2003). Hence, the signalling pathways involved in these processes are also continuously active. In the context of the adult animal it has been shown that the Wnt pathway is involved in the head organizer (Hobmayer et al., 2000) (M. Broun, L.G., B. Reinhardt and H.R.B., unpublished), while the BMP5-8 (Reinhardt et al., 2004), Hedgehog (K. Kaloulis, PhD Thesis, University of Geneva, 2000) and an RTK (Bridge et al., 2000) pathways affect axial patterning processes. Hence, not only are these bilaterian pathways present in cnidarians, they appear to have similar functions.

Another set of signalling pathways involves peptides. For example, peptides affect developmental processes in bilaterians, such as cell proliferation and/or differentiation [e.g. vasopressin (Naro et al., 1997), vasoactive intestinal peptide (Gressens et al., 1997), substance P (Kishi et al., 1996) and gastrin-releasing peptide (Jensen et al., 2001)] or morphogenesis [e.g. bombesin (Sunday et al., 1993), parathyrod hormone-related peptide (Weir et al., 1996)]. None of these peptides has been isolated from hydra so far. However,
several novel peptides have been isolated from hydra and other cnidarians. More recently, a systematic effort has yielded a large number of novel peptides (Takahashi et al., 1997), which are currently being characterized.

Of the peptides characterized so far, five affect patterning processes. Two of them, the Head Activator (Schaller, 1973; Bodennuller and Schaller, 1981) and Heady (Lohmann and Bosch, 2000), are involved in head formation as well as in the initiation of bud formation (Hobmayer et al., 1997). The other three: pedin (Hoffmeister, 1996) pedibin/Hym-346 (Hoffmeister, 1996; Grens et al., 1999) and Hym-323 (Harafuji et al., 2001) promote foot formation. So far the peptides shown to affect patterning processes affect the formation of either the head or foot as a whole. Here, we present evidence that a novel peptide, Hym-301, which was isolated as part of the systematic effort described above (Takahashi et al., 1997) plays a role in a specific part of the head, namely in determining the number of tentacles formed.

Materials and methods

Hydra culture

105, the standard wild-type strain of Hydra magnipapillata (Sugiyama and Fujisawa, 1977), was used for most experiments, while the Basel strain of Hydra vulgaris (Hassel et al., 1993) was used for some experiments. Hydra were cultured as described previously (Sugiyama and Fujisawa, 1977; Martinez et al., 1997). In some experiments, epithelial hydra derived from the 105 strain, which are devoid cells of the interstitial cell lineage except for gland cells (Sugiyama and Fujisawa, 1978) were used. Epithelial hydra were cultured according to Nishimiya-Fujisawa and Sugiyama (Nishimiya-Fujisawa and Sugiyama, 1993).

Isolation and characterization of the gene encoding Hym-301

A full-length cDNA encoding Hym-301 was obtained in three steps. A short cDNA encoding most of Hym-301 was obtained by PCR. Total RNA isolated from budding polyps was used as a template for the synthesis of first strand cDNA using a specific kit (Pharmacia) for this purpose. PCR was carried out using the first strand cDNA, degenerate primers and Taq DNA polymerase (Boehringer Mannheim) for 30 cycles of 94°C for 15 seconds, 50°C for 30 seconds and 68°C for 30 seconds. The fully degenerate primers used corresponded to the amino acids 3-8 (PRRCYL) of Hym-301, and a complementary primer corresponding to the amino acids 9-14 (NGYCSP). The PCR products were separated on a 1.5% agarose gel; the region slightly above the primers was isolated and the DNA isolated with a Mermaid kit (BIO 101). Subsequently, the DNA region slightly above the primers was isolated and the DNA corresponding to the amino acids 3-8 (PRRCYL) of Hym-301, and a degenerate primer corresponding to the amino acids 5-10 of Hym-301 (5’-CAGCACTGACCCCTTTTGT-3’ and AMV reverse transcriptase (Boehringer Mannheim). PCR was carried out using the cDNA, a primer corresponding to the amino acids 5-10 of Hym-301 (5’-AGATGCTACCTTTGTG-3’), a downstream primer of M13 sequence and Taq DNA polymerase for 30 cycles as described above. The amplified DNA was separated on a 1% agarose gel and a 200 bp fragment was detected. The fragment was purified using a GENECLEAN II (BIO 101) kit and cloned into the pCR 2.1 plasmid.

(3) This partial Hym-301 cDNA sequence was used to screen a Hydra cDNA library constructed in Uni-ZapII (Stratagene) (Yum et al., 1998). Screening the library yielded a single clone containing the complete open reading frame was obtained (DDBJ Accession Number: AB106883).

In situ hybridization

Digoxigenin-labelled antisense and sense probes derived from the full-length Hym-301 cDNA precursor sequence were synthesized using an RNA in vitro transcription kit (Boehringer Mannheim). Whole-mount in situ hybridization was carried out as described previously (Grens et al., 1996). Samples were hybridized for 48 hours using a probe concentration of 0.1 ng/ml, and then stained with BM-purple (Boehringer Mannheim) at 37°C for 1 hour in the dark. Thereafter, they were rinsed and incubated in 100% ethanol and subsequently mounted in Euparal (Asco Laboratories).

RNAi

Hym-301 dsRNA and luciferase dsRNA were synthesized as described by Fire et al. (Fire et al., 1998). Both dsRNAs were introduced into stage 2-3 developing buds with localized electroporation (LEP) as described previously (Smith et al., 2000). They were also introduced into regenerating heads as follows. Animals were bisected directly beneath the head, allowed to regenerate for 24 hours and then mounted on glass rods. To do this, the foot and the body column were first bisected parallel to the body axis. Then, a glass needle inserted and threaded up through the gastric cavity to the regenerating head. The other end of the needle was inserted into an agar layer in a petri dish. Then, the LEP procedure was carried out introducing the Hym-301 dsRNA or luciferase dsRNA into the regenerating head from the apical end. The electroporation conditions and dsRNA concentrations were the same as used for introducing the dsRNA into developing buds.

The effect of Hym-301 dsRNA on the level of Hym-301 mRNA in buds was analyzed by RT-PCR as described previously (Technau and Bode, 1999). Tissue was collected from developing buds for each sample that had been treated with LEP. Total RNA was isolated from each sample, and RT-PCR was carried out using primers for the Hym-301 gene (forward, 5’-TTTGCACCTTTATGCGCGA-3’; reverse, 5’-ACCAGGAAACAAATTTTACAT-3’). The hydra EF1α gene was used as an internal control (Technau and Bode, 1999). PCR conditions were as follows: 1 cycle at 94°C for 3 minutes followed by 35 cycles of the following steps: 30 seconds at 94°C, 30 seconds at 50°C and 1 minute at 72°C.

Tissue manipulations

Head and foot regeneration

Head and foot regeneration experiments were carried out using non-budding polyps of the 105 strain of H. magnipapillata. The polyps were bisected in the middle of the body column, and the upper and lower halves allowed to regenerate a foot and head respectively in the presence or absence of 10^-6 M Hym-301. The culture solution with or without the peptide was replaced daily. To assay head regeneration, two methods were used: (1) the number of tentacles formed was counted daily; and (2) whether a mouth had formed was examined daily by treating regenerates with glutathione (GSH at 10^-5 M) for 20 minutes (Lenhoff, 1961). Treated polyps were washed with the culture solution and allowed to continue regeneration. Foot regeneration was assayed by determining if an animal was attached to the floor of the culture dish in which the animals were undergoing regeneration.

LiCl treatment

Non-budding adults of the Basel strain of H. vulgaris were exposed to 2 mM LiCl in hydra medium for 2 days, and then returned to hydra medium.

Measurement of the labelling index

Periodically after bisection, regenerating animals were injected with 5 mM BrdU, and the labelling index of the epithelial cells was
Results

Isolation of the gene encoding Hym-301

The Hym-301 peptide is composed of 14 amino acid residues (KPPRCYLYNGCSP amidase) with one intramolecular disulfide bond and a C-terminal amidation (Takahashi et al., 1997). One clone containing the entire open reading frame of a precursor protein containing Hym-301 as well as the 3′UTR was isolated as described in Materials and methods. Five other Hym-301 cDNA clones have been reported to GenBank, indicating that a complete cDNA is ~660 bp in length including a splice leader sequence (Stover and Steele, 2001). Consistent with these findings, Northern blot analysis indicated the presence of a single Hym-301 transcript about 600 bp in length (data not shown). The cDNA sequence and the deduced amino acid sequence of the precursor protein are shown in Fig. 1. The coding region contains a signal sequence in the N-terminal region and a single copy of the unprocessed Hym-301 near the C terminus. A typical dibasic amino acid (KK) processing site occurs at both ends of the peptide. As Darmer et al. (Darmer et al., 1998) have shown, threonine is often involved in the processing site in cnidarians. Thus, it is plausible that removal of the glutamic acid and threonine at the N-terminal end of the peptide would occur after cleavage at the lysine-lysine site. At the C terminus there is a typical sequence for amidation (glycine followed by dibasic amino acids:GKK) in which the glycine residue serves as an amide donor, while the two lysines serve as the processing site (Sossin et al., 1989).

Hym-301 plays a role in head formation in hydra

To determine where Hym-301 is expressed, in situ hybridization was carried out on whole mounts. The head of a hydra is composed of two parts: the hypostome, or mouth region, in the apical half; and the tentacle zone from which the tentacles emerge in the basal half. Hym-301 is expressed in the tentacle zone, but not the tentacles (Fig. 2). It is also expressed in the hypostome (Fig. 2), although expression in the apical tip of the hypostome is often lower (Figs 3 and 4). The expression of the gene extends down into the apical tenth of the body column, but nowhere else in the adult animal. The gene is clearly expressed in the ectoderm, but not the endoderm. The latter point was demonstrated by carrying out in situ hybridization on animals cut in half longitudinally, which indicated Hym-301 was not expressed in the endoderm (data not shown).

Because of the tissue dynamics of an adult hydra, tissue of the upper body column is continuously converted into tissue of the head (Campbell, 1967). Thus, the expression pattern of Hym-301 suggests the gene plays a role in head formation. As de novo head formation occurs during bud formation and head regeneration, the pattern of expression of the gene was examined in these two developmental processes. Bud formation, the mechanism of asexual reproduction in hydra, begins with an evagination in the lower half of the body column. The evagination elongates into a cylindrical protrusion, which subsequently forms a head at the apical end, a foot at the basal end and detaches. Initiation of head formation is known to begin at the very early stages of bud formation (e.g. Technau and Bode, 1999; Hobmayr et al., 2000). Hym-301 expression first appears once evagination has started (Fig. 3B), but not before (Fig. 3A). As the evagination elongates into the protrusion, expression spreads throughout most of the bud (Fig. 3C-F), although it is absent at the apical tip. Later, when a morphological head becomes visible in terms of tentacles and hypostome (Fig. 3G,H), the expression becomes increasingly restricted to the apical end. When tentacles emerge and elongate, the expression is reduced and later ceases in the tentacles as the pattern takes on the form seen in the adult (Fig. 3G,H).

When a hydra is bisected in the body column, a head regenerates at the apical end of the lower half in 3-4 days, while a foot regenerates at the basal end of the upper half in 1-2 days. The pattern of expression at the apical end of the lower half is similar to that observed during bud formation. 6 hours after bisection, there is very little expression (Fig. 4A). By 12 hours (Fig. 4B) and with increasing intensity over 2 days (Fig. 4C,D), the gene is expressed in the upper half of the regenerate. Then as the tentacles begin to appear by 72 hours (Fig. 4E,F) and elongate by 96 hours (Fig. 4F), expression vanishes in the emerging tentacles, and becomes increasingly restricted to the upper end of the body column. Expression is also absent from the apical tip of the developing hypostome (Fig. 4E,F). Hym-301 was not expressed during foot regeneration (data not shown).
shown), which is consistent with the absence of its expression in the adult (Fig. 2A).

**Hym-301 affects the number of tentacles formed**

The expression patterns indicates the gene could play a role in tentacle formation and in hypostome formation. To determine if the gene affected tentacle formation, animals were bisected in the middle of the body column and the basal halves allowed to regenerate heads in the presence or absence of the Hym-301 peptide. As the tentacles form in a regenerating head in 4-6 days, regenerates were examined daily for 7 days. Exposure to $10^{-8}$ M or $10^{-7}$ M peptide had no effect. However, treatment with either $10^{-6}$ M or $10^{-5}$ M resulted in an increase in the number of tentacles that formed (Fig. 5A). As the number of tentacles formed is correlated with the diameter of the tentacle zone (Bode and Bode, 1984), Hym-301 might affect the rate of epithelial cell division in the developing head. This possibility was assayed by bisecting animals in the middle of the body column, exposing them to $10^{-6}$ M Hym-301 for 3 days. Daily, the apical quarter as well as the next quarter was isolated from control and from peptide-treated regenerating animals, and the labelling index of the epithelial cells measured. As shown in Table 1, the peptide had no significant effect on the labelling index in either region, and hence, the rate of cell division. Thus, the increase in tentacle number is not due to an increase in the number of epithelial cells in the regenerating head. The peptide also had no effect on the rate of tentacle formation nor on the final length of the tentacles (data not shown).

Another assay for the role of the gene in tentacle formation involved the use of RNAi (Fire et al., 1998) to block the activity of the gene. Hym-301 dsRNA was synthesized and introduced into young buds (stage 2-3) using a localized electroporation procedure (Smith et al., 2000). Subsequently the number of tentacles was determined in the developing buds. As shown in Fig. 6, the number of tentacles formed in buds treated with Hym-301 dsRNA was significantly lower than in untreated controls. And, it remained lower as there was no significant increase between 7 and 15 days after introduction of the Hym-301 dsRNA (data not shown). The number was also lower than in buds in which luciferase dsRNA had been introduced to control for the electroporation procedure as well as the introduction of dsRNA. In a second experiment, the two types of dsRNA were introduced into regenerating heads. Animals were bisected at the head/body column border, allowed to regenerate for one day so that the tissue at the apical end would stretch over the gastric cavity and form a dome. Then, using a modification of the localized electroporation as described in the Materials and methods, either of the two dsRNAs was introduced into the regenerating heads. The number of tentacles formed was determined 7 days later. As shown in Table 2, the number of tentacles formed in regenerating heads into which Hym-301 dsRNA was introduced was smaller than in those receiving luciferase dsRNA. The introduction of Hym-301 dsRNA into developing buds also transiently reduced the level of Hym-301 RNA in the developing buds for at least 12 days.

### Table 1. Effect of Hym-301 on the epithelial cell labelling index

<table>
<thead>
<tr>
<th>Region</th>
<th>Length of treatment (days)</th>
<th>Labelling index (%)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Control</td>
<td>Hym-301</td>
<td></td>
</tr>
<tr>
<td>Apical quarter</td>
<td>1</td>
<td>20.0±5.4</td>
<td>21.1±4.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>31.1±6.8</td>
<td>36.8±3.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>27.7±3.0</td>
<td>25.5±4.3</td>
<td></td>
</tr>
<tr>
<td>Next quarter</td>
<td>1</td>
<td>22.6±6.2</td>
<td>21.9±5.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>23.9±4.1</td>
<td>23.6±2.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>15.6±1.2</td>
<td>17.8±4.2</td>
<td></td>
</tr>
</tbody>
</table>

Each value is the average value±s.d. for three measurements. Two-hundred epithelial cells were counted for each measurement.

**Fig. 3.** Expression of Hym-301 gene (dark blue) during the stage of bud formation. (A) Stage 0, (B) stage 1-2, (C) stage 2-3, (D) stage 3, (E) stage 4, (F) stage 5, (G) stage 6-7 and (H) stage 8.

**Fig. 4.** Expression of Hym-301 gene (dark blue) during head regeneration. (A) Six hours, (B) 12 hours, (C) 24 hours, (D) 48 hours, (E) 72 hours and (F) 96 hours.
Hym-301 affects tentacle formation and up to about 24 hours (Fig. 7). This reduced level of the Hym-301 RNA is correlated with the delayed appearance of the tentacles (Fig. 6) as well as with the lowered number of tentacles that are formed. Thus, Hym-301 clearly plays a role in tentacle formation.

Treatment of bisected animals with the peptide had no obvious effect on hypostome development during head regeneration. To determine if the peptide affected the rate of hypostome formation regenerates were assayed periodically for mouth formation. The mouth of a hydra is in the apical part of the hypostome. When an adult animal is exposed to $10^{-5}$ M glutathione, it opens its mouth widely within 10 minutes (Lenhoff, 1961). During normal head regeneration, periodic exposure to this reagent indicates that a mouth begins to form within 3-4 days of decapitation, and is fully formed by 7 days (Fig. 5B). The rate of mouth formation was no different in regenerates exposed to the peptide indicating that it had no effect on the rate of mouth formation (Fig. 5B).

As the major effect of treatment with the Hym-301 peptide treatment or RNAi was the alteration in the number of tentacles formed, the gene appears to be required for the regulation of the number of tentacles formed.

**Hym-301 directly acts on epithelial cells**

Hym-301 is produced in ectodermal epithelial cells. What is/are the target cell(s)? Hydra has three cell lineages: the ectodermal epithelial cell, endodermal epithelial cell and interstitial cell lineage. The latter consists of a multipotent stem cell and several classes of differentiation products (Bode, 1996). Hym-301 could act directly on epithelial cells of both the ectoderm and endoderm to form tentacles, or it could act indirectly through a cell type of the interstitial cell lineage, such as the neurons. To determine if cells of the interstitial cell lineage are involved, epithelial animals, which are devoid of cells of the interstitial cell lineage (Sugiyama and Fujisawa, 1978), were bisected in the middle of the body column and allowed to regenerate in the presence or absence of $10^{-6}$ M Hym-301. As epithelial animals have somewhat larger heads, which regenerate more slowly, the analysis was carried out after 10 days instead of 7 days for the normal animals. The larger number of tentacles in the epithelial animals is also a reflection of the larger head size of these animals compared with normal animals of this species of hydra. As shown in Table 3, Hym-301 significantly increased the number of tentacles formed in the epithelial hydra, indicating that the

![Fig. 5.](image)

Effect of Hym-301 on the number of tentacles formed (A), and the rate of hypostome formation (B) during head regeneration. Both figures represent regenerates treated with the Hym-301 peptide (black circles) and controls (white circles). Each data point is the average value±s.e.m. for three experiments. Fifteen to 20 regenerates were used in each of the experiments.

**Table 2. Effect of Hym-301 dsRNA on the number of tentacles formed in regenerating heads**

<table>
<thead>
<tr>
<th>Source of dsRNA</th>
<th>Number of samples</th>
<th>Number of tentacles formed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hym-301</td>
<td>122</td>
<td>3.78±0.08</td>
</tr>
<tr>
<td>Luciferase</td>
<td>117</td>
<td>4.25±0.09</td>
</tr>
</tbody>
</table>

Each value is the average value±s.e.m. for five experiments. Twenty to 25 regenerates were used for each sample in each experiment. The number of tentacles was assayed 7 days after decapitation.

**Table 3. Effect of Hym-301 on the number of tentacles formed in regenerating head of epithelial animals**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of samples</th>
<th>Number of tentacles formed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hym-301</td>
<td>85</td>
<td>9.0±0.1</td>
</tr>
<tr>
<td>Control</td>
<td>81</td>
<td>8.2±0.2</td>
</tr>
</tbody>
</table>

Each value is the average value±s.e.m. for six experiments. Ten to 15 regenerates were used for each sample in each experiment. The number of tentacles formed was assayed 10 days after introduction of the dsRNA.

![Fig. 6.](image)

Effect of Hym-301 dsRNA on the number of tentacles formed during bud formation: Hym-301 dsRNA-treated buds (black circles), Luciferase dsRNA-treated buds (triangles) and untreated control buds (white circles). Each data point is the average value±s.e.m. for five experiments. In each experiment, 10-20 animals were used.
peptide acts directly on the epithelial cells. Hence, the cells of the interstitial cell lineage are not required for the activity of Hym-301.

**Hym-301 expression is regulated by the head activation gradient**

A major factor controlling axial patterning in hydra is a morphogenetic gradient variously referred to as a positional value gradient (Wolpert, 1971), a source density gradient (Gierer and Meinhardt, 1972) or a head activation gradient (MacWilliams, 1983). The expression patterns of other genes, which are normally expressed in a specific area along the body axis are altered when the head activation gradient is either raised [e.g. Cnox-2 (Shenk et al., 1993)] or lowered [e.g. CnNK2 (Grens et al., 1996), BMP5-7b (Reinhardt et al., 2004)]. Because Hym-301 is expressed only in the upper one-tenth of the body column and in the head, it may be expressed above a threshold value of the gradient. Thus, altering the gradient could alter the range of expression of Hym-301 along the column.

When hydra are treated with 2 mM LiCl, the level of the head activation gradient is raised throughout the body column. This was demonstrated by transplanting pieces isolated from different levels of the body column tissue of a donor treated with 2 mM LiCl for 2 days to untreated hosts at the same axial level. A higher fraction of the Li⁺-treated transplants for each axial level formed second axes than did the corresponding control region, indicating a rise in the head activation level in the Li⁺-treated tissue (L.G. and H.R.B., unpublished). Furthermore, this treatment invariably leads to the formation of ectopic tentacles form along the body column (Hassel and Bieler, 1996). Hydra were exposed to 2 mM LiCl and the expression pattern of Hym-301 analyzed at various times after begin of treatment. As shown in Fig. 8, the pattern of expression extends further down the body column with increasing time of exposure to LiCl. As the gradient rises throughout the body column during the exposure to 2 mM LiCl, the threshold level for Hym-301 expression would move down the column, which is consistent with the observed changes in expression of the gene. In turn, this results indicates that Hym-301 expression is regulated by the head activation gradient.

**Discussion**

**Tentacle formation in hydra**

Tentacle formation occurs in three steps: (1) the specification of body column tissue for tentacle formation; (2) the commitment of spots of tentacle zone tissue for tentacle formation; and (3) the evagination of the spots to form tentacles coupled with the differentiation of the epithelial cells. The evidence for these steps has been gained by examining the formation of tentacles in a developing head during bud formation or head regeneration, and during the continuous displacement of tissue from the body column onto tentacles in adult hydra.

In an adult hydra, the epithelial cells of both tissue layers in the body column are constantly in the mitotic cycle (David and Campbell, 1972; Campbell and David, 1974). A consequence of this activity is that tissue of the upper body column is continuously displaced into the head and sloughed at the extremities. In the rest of the body column, tissue is displaced down the axis onto developing buds, which eventually detach, or into the foot and sloughed (Campbell, 1967). Thus, the animal remains constant in size as it is in a steady state of production and loss of cells.

The first step, the specification of body column tissue for tentacle formation takes place within the context of the processes governing axial patterning. The head organizer in the hypostome (Broun and Bode, 2002) produces and transmits a signal to the body column that sets up a morphogenetic gradient, the head activation gradient, which is maximal in the hypostome and decreases down the body column. Different values of the gradient define the different regions: hypostome, tentacle zone, body column and foot. Because of the tissue dynamics, these processes are constantly active.

As tissue is displaced up the body column, it moves up the head activation gradient, and when it crosses a threshold value it becomes specified for tentacle formation. The evidence for this is the following. Tissue of most of the body column is capable of forming either a head or a foot. However, tissue in the upper one-eighth of the body column depending on the
species of hydra is no longer capable of foot formation (L.G. and H.R.B., unpublished). As shown by Hobmayer et al. (Hobmayer et al., 1991), this tissue as well as that in the tentacle zone has become specified/committed to tentacle formation. In the head, this commitment is confined to the lower part of the head, the tentacle zone, as tissue more apically in the hypostome does not form tentacles.

The second step involves commitment of tissue in the tentacle zone to the formation of individual tentacles. During bud formation or head regeneration, this occurs in evenly spaced spots located around the developing tentacle zone, as illustrated with two molecular markers. When a developing bud has elongated into a cylindrical shell, both HyAlx and HyBMP5-8b, hydra orthologues of aristaless and BMP5-8, are expressed in a ring of spots below the apical tip of the bud (Smith et al., 2000; Reinhardt et al., 2004). The same sequence of steps occurs during head regeneration. The spots are evenly spaced, suggesting the involvement of a mechanism that sets up a spacing pattern. This view is supported by the following observation. Increasing the diameter of a regenerating head by threading a thin glass needle through the gastric cavity following bisection results in the formation of more tentacles than in simply bisected animals (Bode and Bode, 1984).

After the formation of the pattern of spots, each spot evacinates and elongates into a tentacle as tissue from the tentacle zone is displaced onto each tentacle. This is the third step. As a tentacle evacinates and elongates, HyAlx and HyBMP5-8b continue to be expressed as rings at the tentacle zone/tentacle border (Smith et al., 2000; Reinhardt et al., 2004). They mark a sharp transition in the activity of cells moving across the tentacle zone/tentacle border.

As cells are displaced through the tentacle zone, they remain in the mitotic cycle until they cross the tentacle/tentacle zone border onto tentacles. Then, they abruptly cease dividing and differentiate (Holstein et al., 1991). The expression patterns of a number of genes also illustrates this sudden change. Cnotx (Smith et al., 1999) and Cnotx-3 (Bode, 2001), the hydra orthologues of Otx and labial/Hox-1 are expressed in epithelial cells of the tentacle zone, but cease expression at the tentacle zone/tentacle border. Conversely, TS-19, a cell surface antigen (Bode et al., 1988), an annexin gene (Schlaepfer et al., 1992), HMP1 a metalloprotease (Yan et al., 1995) and HTK (Steele et al., 1996) are expressed immediately as the epithelial cells cross the tentacle/tentacle zone border and subsequently all along the tentacles.

Most likely, the two genes expressed at the border, HyAlx and HyBMP5-8b play a role in the differentiation of cells as they cross the border, or in the change in cell shape to convert tentacle zone tissue into tentacle tissue. Transiently reducing the level of HyAlx mRNA in a developing bud with RNAi leads to a delay in tentacle formation, which supports this view (Smith et al., 2000).

**Hym-301 affects the number of tentacles formed**

Several results indicate that Hym-301 may play a role in the first step, and clearly plays a role in the second step of tentacle formation.

A role in specification of tissue for tentacle formation

As tissue is displaced up the body column, the level of head activation in the tissue rises passing a threshold for commitment of tissue for tentacle formation. Two results indicate that Hym-301 expression occurs above this threshold level.

1. In the normal animal the gene is expressed in the upper one-eighth of the body column, the tentacle zone and the hypostome. Tissue in this part of the body column and the tentacle zone is specified/committed to tentacle formation (Hobmayer et al., 1990).

2. In LiCl-treated animals, the head activation gradient is raised throughout the body column (L.G. and H.R.B., unpublished). This rise is correlated with Hym-301 expression being displaced down the body column. And, the longer the treatment with LiCl, the further down the body column the gene was expressed. This rise in head activation was also reflected in the formation of ectopic tentacles along the body column. Thus, there is a strong correlation between Hym-301 expression and tentacle formation.

However, it is unlikely that the Hym-301 peptide commits tissue to tentacle formation. The gene is also expressed in the hypostome, where tentacles do not form. In addition, during early stages of bud formation and head regeneration, Hym-301 is expressed throughout much of a developing bud and the upper half of the body column of a hydra regenerating a head where tentacles will not form. Instead, these results are consistent with the expression of the gene above a threshold level of the head activation gradient. A plausible explanation for these results is that the Hym-301 peptide is involved in specifying, but not committing epithelial tissue to tentacle formation. In tissue that will develop into a hypostome, or that is in the hypostome, additional factors required for commitment to tentacle formation are not present. Or, other molecular mechanisms may override tentacle specification.

**Role in the number of tentacles formed**

Hym-301 has a clear role in the second step: the determination of the number of tentacles formed in a developing head. Addition of the peptide to a regenerating head results in an increase in the number of tentacles formed. Reduction of the level of the peptide with RNAi during bud formation or head regeneration leads to a reduction in the number of tentacles formed. As in normal development, the tentacles formed are evenly spaced after these treatments. This indicates that a mechanism generating a spacing pattern is involved in the process of tentacle formation, and that the Hym-301 peptide may play a role in this mechanism.

Assuming a spacing pattern is involved, an increase in the number of tentacles could be the result of an increase in the diameter of the tentacle zone where the tentacles will form. If so, one would expect an increase in the number of epithelial cells in the developing tentacle zone. As treatment of epithelial animals (animals consisting only of epithelial cells) with the Hym-301 peptide also results in an increase in the number of tentacles formed, the peptide clearly can act directly on epithelial cells. However, treatment with the peptide did not lead to an increase in the rate of cell division, or in the number of epithelial cells in a regenerating head. Thus, the peptide must affect the spacing pattern in some other manner.

The two roles of Hym-301 could be part of a single mechanism

Gierer and Meinhardt (Gierer and Meinhardt, 1972)
constructed a model based on a reaction-diffusion mechanism to explain axial patterning in hydra. The essence of the model is that an autocatalytic activator rises to a threshold level committing the tissue to a particular development: for example, head formation. At the same time, the activator induces the production of an inhibitor which diffuses to prevent formation of another activator peak in the vicinity.

Meinhardt (Meinhardt, 1993) refined the model by considering the formation of the tentacles and the hypostome/head organizer to be based on separate reaction-diffusion mechanisms. The mechanism for tentacle formation is activated above a threshold value of head activation, which would be in the tentacle zone. At a higher level of positional value in the prospective hypostome, the activator/inhibitor mechanism for hypostome/head organizer would be activated, which in turn blocks tentacle activation. This confines tentacle activation to a ring of tissue, the tentacle zone beneath the hypostome.

The mechanism for tentacle formation involves the autocatalytic rise of tentacle activation (TA) coupled with the activator-induced production of a tentacle inhibitor (TI). As the rise in tentacle activation around the tentacle zone is unlikely to be uniform, those spots of TA which started earlier will produce more TI, and block the rise of tentacle activation in their neighbourhood. Such a mechanism will result in a fairly evenly spaced pattern of TA peaks – each of which will eventually form a tentacle.

As Hym-301 is expressed throughout the tentacle zone, it may play a role in the tentacle activation process. Then, external addition of peptide would raise the overall concentration of the peptide in the tentacle zone causing the more rapid rise of the TA throughout the tentacle zone. In turn, this would result in more TA peaks reaching the level where commitment to tentacle formation occurs before sufficient amounts of tentacle inhibitor are produced by developing neighbouring peaks. This would result in a spacing pattern with more tentacles. Conversely, if the amount of Hym-301 peptide is lower than normal, the rise in TA would be slower, and fewer peaks would reach the level where they are resistant to the continuously produced tentacle inhibition. This would result in a spacing pattern with fewer tentacles.

Such a mechanism could account for both of the activities proposed for the Hym-301 peptide proposed in the previous section. First, the peptide would have a role in specifying tissue for tentacle formation in that it is part of the tentacle activation process. And second, it would have a role in the number of tentacles formed as it affects the rate of rise in a TA peak. The final number of tentacles formed is a function of this rate or TA rise.

Role of peptides in axial patterning in hydra

A number of peptides have been isolated from hydra and shown to participate in axial patterning processes. As mentioned earlier, three of them, pedin (Hoffmeister, 1996), pedibin/Hym-346 (Hoffmeister, 1996) and Hym-323 (Harafugi et al., 2001), affect the rate of foot regeneration, while the head activator increases the rate of head as well as foot regeneration (Schaller, 1973; Javois and Tombe, 1991; Javois and Frazier-Edwards, 1991). More direct roles have been identified for these two peptides, as well as for a third. Both pedibin/Hym-346 (Grens et al., 1996) and Hym-323 (Harafugi et al., 2001) lower the head activation gradient, while Heady appears to be involved in the development of the head organizer (Lohman and Bosch, 2000). In this regard, Hym-301 is similar to the latter group as it affects a specific patterning event.

Whether all five of the peptides directly affect the epithelial cells of the two layers, the ectoderm and endoderm, which carry out the patterning processes is not clear. The head activator, pedin and pedibin/Hym-346 also affect the rates of cell division and neuron differentiation (Schaller et al., 1989; Hoffmeister, 1996). Thus, it is plausible that these peptides affect patterning processes indirectly. By contrast, Hym-323 (Harafugi et al., 2001) and Hym-301 have been shown to have similar effects on epithelial animals as they do on normal hydra. As the epithelial animals consist only of the epithelial cells of the two tissue layers, these peptides affect the epithelial cells directly.

Of the common signalling pathways that affect developmental processes in bilaterians, three of them are known to affect patterning processes in hydra. The Wnt pathway is involved in the formation of the head organizer (Hobmayer et al., 2000) (M. Broun, L.G., B. Reinhardt and H.R.B., unpublished), while the members of the RTK and BMP pathways are involved in the patterning of the lower part of the body column (Steele et al., 1996; Reinhardt et al., 2004), which involves the head activation gradient. Heady may also affect the organizer (Lohmann and Bosch, 2000), while both pedibin/Hym-346 and Hym-323 (Grens et al., 1996; Harafugi et al., 2001) can lower the head activation gradient. Hence, it appears as though the well-established signalling pathways as well as pathways initiated by some of these peptides may be involved, and possibly interacting, in these patterning processes.

This raises the issue of whether this is peculiar to hydra, or whether it also occurs in bilaterians. That one of these peptides also plays a role in bilaterians has been shown for the head activator. This peptide has also been isolated from the hypothalamus and intestine of rats and humans where it also plays a role in bilaterians has been shown for the head activator (Schaller, 1975; Bodenmüller et al., 1980; Bodenmüller and Schaller, 1981). Thus, it is plausible that such peptides represent a class of signalling molecules that have been initially uncovered in hydra, and may also exist in bilaterians.

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