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The novel signal peptides, Pedibin and Hym-346, lower positional value thereby enhancing foot formation in hydra

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SUMMARY

Signaling molecules affecting patterning processes are usually proteins and rarely peptides. Two novel peptides, pedibin and Hym-346, that are closely related to one another have been isolated from *Hydra vulgaris* and *Hydra magnipapillata*. Several experiments indicate that both cause a reduction in the positional value gradient, the principle patterning process governing the maintenance of form in the adult hydra. The peptides cause an increase in the rate of foot regeneration following bisection of the body column. Treatment of animals with either peptide for an extended period of time resulted in an apical extension of the range of expression of *CnNk-2* along the body column. Such an extension is correlated with a decrease in positional value. Transplantation of tissue treated with Hym-346 results in an increase in the fraction forming feet, and aggregates derived from Hym-346 tissue form more feet and fewer heads. The latter two experiments provide a direct measure of the lowering of positional value in the treated tissue. These results suggest that peptides play signaling roles in patterning processes in cnidaria and, plausibly, in more complex metazoans as well.

Key words: Hydra, Pedibin/Hym-346, Foot formation

INTRODUCTION

Commonly the signaling molecules regulating pattern-forming processes in animals have been shown to be proteins, some of which are members of the Hedgehog, Wingless or TGF-β familes. In contrast, peptides (as well as proteins) are known to play roles in other developmental processes 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) or morphogenesis (e.g. bombesin, Sunday et al., 1993; parathyroid hormone-related peptide, Weir et al., 1996). Whether peptides are also involved in patterning processes is unclear.

There is some evidence suggesting that peptides affect patterning processes in cnidaria. Members of the LWamide family of peptides isolated from the sea anemone, *A. elegantissima* (Leitz et al., 1994) and hydra (Takahashi et al., 1997) have been shown to induce the metamorphosis of larvae into polyps in hydractinia (Leitz et al., 1994; Takahashi et al., 1997). Three other peptides affect developmental processes in hydra. Head activator is known to increase the rate of head regeneration (Schaller, 1973; Javois and Tombe, 1991) as well as increase the rate of budding, the animal's asexual form of reproduction (Schaller, 1973; Hobmeyer et al., 1997). More recently, Hoffmeister (1996) has shown that two other peptides, pedibin and pedin, increase the rate of foot regeneration. In addition, pedin increases the rate of interstitial cell proliferation and neuron differentiation (Hoffmeister, 1996).

Because the epithelial cells of both cell layers are constantly in the mitotic cycle (David and Campbell, 1972; Campbell and David, 1974), all cells in the adult hydra are constantly changing location (Campbell, 1967). Hence, patterning processes are constantly active to maintain the form of the animal as well as the regional distributions of cells. Since these patterning processes are well understood at a cell and tissue level (e.g. Bode and Bode, 1984a), hydra provides a useful system for examining the role of these, or other, peptides in a patterning process. The principle manifestation of these processes is a positional value gradient that is maximal in the head and decreases down the body column towards the foot (Wolpert et al., 1971; Müller, 1996). A high positional value leads to head formation, while a low one results in foot formation. The positional value at any location can be raised (Müller, 1990) by treating animals with diacylglycerol, which eventually leads to the formation of ectopic heads in the body column (Müller, 1989), or lowered by treating with LiCl (Maggiore and H. R. B., unpublished results), which results in the formation of ectopic feet (Hassel and Berking, 1990). These results suggest the involvement of the phosphatidylinositol cycle in the patterning process.

However, little else is known about the molecular basis of the gradient.

Here we present evidence that two closely related peptides, pedibin isolated from *Hydra vulgaris* (Hoffmeister, 1996) and Hym-346 isolated from *Hydra magnipapillata* (Takahashi et al., 1997) play a role in this positional value gradient. Both peptides lower the positional value as measured by an increase in the ability of tissue of the body column to form feet in transplantation and aggregation experiments as well as changes in the expression pattern of a gene whose expression is closely coupled with the positional value gradient. These results provide the first direct evidence for the involvement of a peptide, as opposed to a protein, as a signaling molecule in a defined patterning process.

MATERIALS AND METHODS

Hydra and peptides

Two species of hydra, the 105 strain of *Hydra magnipapillata* and the Basel strain of *Hydra vulgaris*, were used for almost all of the work presented here. For one experiment, the L2 strain of *Hydra vulgaris* was used. All three strains were maintained as previously described (*H. magnipapillata* as in Takano and Sugiyama, 1983; *H. vulgaris* as in Grens et al., 1996). Animals were fed either daily (*H. magnipapillata)* or twice a week (*H. vulgaris)*, and the medium changed daily. Synthetic peptides corresponding to pedibin and Hym-346 described previously (Hoffmeister, 1996; Takahashi et al., 1997) were used for all experiments. Specific fragments of Hym-346 described in Table 1 were synthesized and kindly provided by Dr Takahashi. Each peptide was dissolved in hydra medium at a concentration of 10^{-6} M, and either used undiluted or diluted as described in individual experiments.

Tissue manipulations

Whole animals or regenerates were exposed to either pedibin or Hym-346 for varying periods of time depending on the experiment as indicated in the Results section. The peptide solution was replaced daily and the animals were fed on a normal schedule. The treated animals were then subjected to one of four different kinds of manipulations.

(1) Foot regeneration

Two kinds of foot regeneration experiments were carried out. In one, non-budding adults of the Basel strain of *Hydra vulgaris* were treated with 10^{-6} M Hym-346 for 48 hours, bisected in the middle of the body column and the upper half allowed to regenerate in the presence of 10^{-6} M Hym-346 for 48 hours. Samples were assayed for foot formation using the peroxidase assay based on the procedure described by Hoffmeister and Schaller (1985). The assay was modified as follows. After bisection animals were fixed at various times in 4% paraformaldehyde in hydra medium for 30-60 minutes. Then, samples were washed 3× for 5 minutes each in 1×PBS containing 0.25% Triton X-100, and subsequently stained with a solution containing 0.5 mg/ml 1,4-phenylenediamine dihydrochloride, 1 mg/ml catechol and 0.02% H_2O_2 in 0.1 M Tris, pH 7.6 for 2 minutes. Thereafter, samples were washed 2× for PBS-Triton as before and dehydrated by rinsing for 2 minutes each in 70%, 95% and 100% ethanol before mounting in Euparal (Carolina Biological Supply).

In the second experiment, adults of the 105 strain of *Hydra magnipapillata* were treated with Hym-346 for 24 hours, the upper peduncle isolated, allowed to regenerate for 24 hours and then stained with AE03 monoclonal antibody (kindly provided by Y. Kobayakawa). This antibody specifically stains the ectodermal epithelial cells of the basal disk (Amano et al., 1997). The immunocytochemistry was carried out as described by Koizumi et al. (1988).

(2) Transplantation experiments

Lateral transplantion to measure changes in the foot-forming potential was carried out as described by Sugiyama (1982) and in Fig. 3. *H. magnipapillata* were treated with 10^{-6} M or 10^{-8} M Hym-346 for 6 days or 15 days, respectively. Thereafter, a region of a treated donor body column was transplanted into a specific location in an untreated host. Budding animals were used as donors and hosts to define the source of tissue in a donor to be transplanted as well as the site of transplantation in the host. The criterion for foot formation was the ability of the distal end of the transplant to trap a bubble of air. A bubble of air trapped on a pair of forceps is transferred to the presumptive foot. If the transfer is successful, a foot has formed. The donor tissue and the site of transplantation for individual experiments are indicated in the Results section.

(3) Aggregation experiments

H. vulgaris animals were treated with 10^{-6} M Hym-346 for 15 days, the body columns isolated and aggregates formed as described previously (Gierer et al., 1972; Technau and Holstein, 1992). Aggregates were allowed to develop for up to 5 days and were treated with 10^{-6} M Hym-346 during that period starting 1 day after aggregates were formed. The one modification of the published procedures was the dilution schedule of the aggregate incubation medium from dissociation medium (DM) to hydra medium (HM) following centrifugation. The dilution was through steps from DM to 50% DM: 50% HM to 25% DM:75% HM to HM at 6 hours, 18 hours and 30 hours, respectively. Periodically, samples of aggregates were analyzed for the expression of *CnNK-2* as described below. A structure or protrusion was considered a head when it had a clear hypostome with at least two tentacles. Feet were defined as patches or protrusions that were *CnNK-2*$^+$.

In situ hybridization

The expression pattern of *CnNK-2* in whole animals or aggregates was determined using a modified version of the whole-mount in situ hybridization procedure described by Grens et al. (1996). The modifications were the following. The concentration of the digoxigenin-labeled probe used in the hybridization step was 6 ng/μl. Instead of the NBT + BCIP stain, the samples were stained with BM-Purple (Boehringer-Mannheim) as follows. After removal of the antibody and the wash steps described previously, the last wash was replaced with 0.5 ml BM-Purple, and samples stained in the dark at 37°C for 1.5 hours. Thereafter, the samples were rinsed with 100% ethanol, incubated in 100% ethanol for 30-120 minutes, followed by a final 100% ethanol rinse and then mounted in Euparal.

RESULTS

Hym-346 increases the rate of foot regeneration

Since the amino acid sequences for pedibin and Hym-346 are identical (see Table 1) except for the additional glutamine at the C-terminal end of pedibin, it is likely that they have similar functions. Pedibin has been shown to affect the rate of foot regeneration (Hoffmeister, 1996). To determine if Hym-346 had a similar effect, the following experiment was carried out. *H. vulgaris* adults were treated with a range of concentrations of Hym-346 for 48 hours and then bisected in the mid-body column. The upper halves were allowed to regenerate for 48 hours in the presence of 10^{-6} M Hym-346, and subsequently assayed for regeneration of a foot using the peroxidase assay

Table 1. Amino acid sequences of peptides used in the described experiments

Peptide	Amino acid sequence
Pedibin	AGEDVSHELEEKEKALANHSE
Hym-346	AGEDVSHELEEKEKALANHS
N-fragment	AGEDVS
F-fragment	HELEEKEK
C-fragment	ALANHS

(Hoffmeister and Schaller, 1985), which was modified as described in Materials and Methods. As shown in Fig. 1, treatment with Hym-346 resulted in an increase in the fraction of animals having regenerated a foot in 48 hours. Further, the size of this fraction was directly correlated with the concentration of the peptide used indicating that the higher the concentration of peptide used, the faster the foot regenerated. Since all of the upper halves will regenerate a foot without treatment, the results indicate that the peptide affects the rate of foot regeneration.

The effect of Hym-346 on foot regeneration was examined also in *H. magnipapillata* with a slightly different method. *H. magnipapillata* adults were treated with a range of concentrations (10^{-9}-10^{-6} M) of Hym-346 for 24 hours. Then, the upper half of the peduncle, the part of the body column between foot and the budding zone was excised, and allowed to regenerate for 24 hours. By assaying the pieces with AE03, a foot-specific monoclonal antibody (Amano et al., 1997), it was shown that the excised pieces derived from animals treated with the peptide regenerated faster than controls (data not shown).

Hence, both pedibin and Hym-346 increase the rate of foot regeneration.

Hym-346 extends the range of *CnNK-2* expression up the body column

The ability of both pedibin and Hym-346 to increase the rate of foot regeneration suggests they play a role in patterning of the body column. Or, more precisely, the peptides may have an effect on the positional value gradient. Three approaches described in this and the following sections were used to gain more direct evidence that the peptides lower the positional value and increase the probability of foot formation.

One makes use of the homeobox gene, *CnNK-2*, which is expressed in the epithelial cells of the endoderm. The expression pattern of this gene is graded up the body column with a maximum in the lower peduncle just above the foot and fading out just above the budding zone (Grens et al., 1996). Since the range of expression along the body axis is altered by treatments, which are known to affect the positional value gradient (Grens et al., 1996), the gene provides a marker for changes in positional value. Two experiments were carried out with this gene.

In one, animals of the Basel strain of *H. vulgaris* were treated with 10^{-6} M Hym-346 for 0, 5, 10 or 15 days, and the expression pattern of *CnNK-2* analyzed on whole mounts using in situ hybridization. As shown in Fig. 2, the full 15 day treatment caused a substantial displacement of the apical border of the range of expression in an apical direction along the axis of the body column. Further, the extent of the apical displacement of this border was directly correlated with the length of the Hym-346 treatment (Table 2). To determine that the effect was not due to a non-specific effect of the high concentration of the peptide, animals were treated with three different fragments of Hym-346, which are described in Table 1, at concentrations of 10^{-6} M using the same conditions as

Table 2. Effect of Hym-346 on the range of *CnNK-2* expression

Peptide used in treatment	Length of treatment (days)	Fraction of body column stained with *CnNK-2* (%)
Hym-346	0	35.2±9.3
Hym-346	5	42.9±9.7
Hym-346	10	47.0±9.4
Hym-346	15	52.8±5.8
control	0	32.5±10.9
fragment N	15	34.2±9.7
fragment F	15	32.7±9.1
fragment C	15	34.6±8.8

Fragments N,F,C of Hym-346 are shown in Table 1. 50 animals were measured for each sample. Error is the standard deviation.

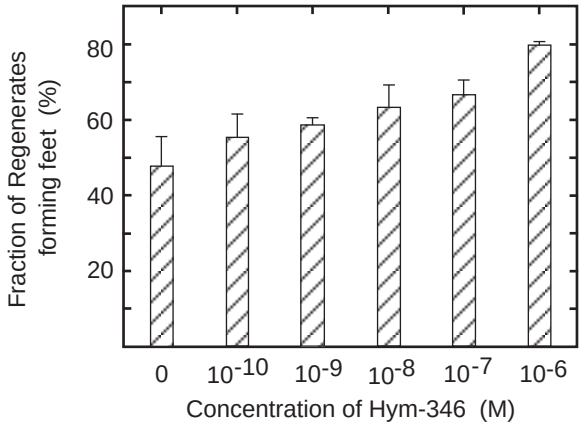

Fig. 1. Effect of Hym-346 on the rate of foot regeneration in *Hydra vulgaris.* The value for each concentration is the average ±s.d. of three separate experiments. In each experiment, each value is the average 30-35 regenerates.

Fig. 2. Pattern of *CnNK-2* expression in *Hydra vulgaris.* (A) A control and (B) an animal treated for 15 days with Hym-346. Scale bar in A (also applies to B) represents 0.5 mm.

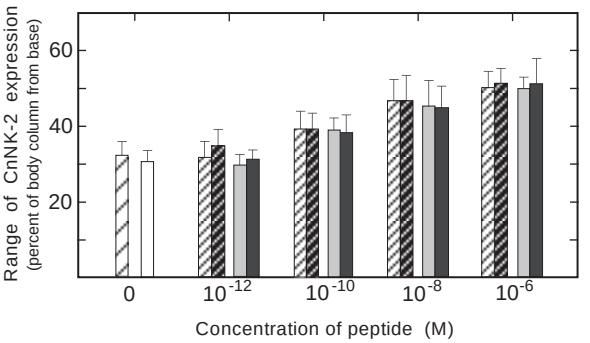

Fig. 3. Effect of increasing concentrations of pedibin or Hym-346 on the apical extension of the *CnNK-2* pattern in *Hydra vulgaris* and *Hydra magnipapillata.* ▨, *Hydra vulgaris*/control; ▨, *Hydra vulgaris*/Hym-346; ▨, *Hydra vulgaris*/pedibin; □, *Hydra magnipapillata*/control; ▨, *Hydra magnipapillata*/Hym-346; ■, *Hydra magnipapillata*/pedibin. Each value is the average ± s.d. of 50 animals.

before. None of these peptide fragments altered the range of expression of *CnNK-2* (Table 2), indicating that the effect of the intact peptide was specific.

Treatment with pedibin had a similar effect. *Hydra magnipapillata* were treated with 10^{-6} M of pedibin for 5, 10 or 15 days. With increasing length of treatment, the apical border of the *CnNK-2* range was displaced in an apical direction (data not shown).

In the second experiment, the effect of concentration of the peptide on the range of *CnNK-2* expression was examined. Adult animals of both the *Hydra vulgaris* and *Hydra magnipapillata* species were treated for 15 days with a range of concentrations of either Hym-346 or pedibin. As shown in Fig. 3, the extent of displacement of the apical border of the pattern of *CnNK-2* expression was directly correlated with the concentration of the peptide. Further, both species were affected equally by either peptide indicating that the single additional amino acid at the C-terminal end of pedibin had no effect on the expression of this gene. Hence, the two peptides, despite a single amino acid change most likely have similar biological effects at similar concentrations.

In both experiments, the range of expression of this gene was extended apically along the body column. Since such an apical extension is correlated with a reduction in positional value along the entire body column, these results indicate that pedibin/Hym-346 causes a decrease in positional value.

Hym-346 increases the probability of foot formation in transplantation experiments

The changes in the *CnNK-2* expression pattern suggests that Hym-346 has lowered the positional value gradient. A more direct means of measuring changes in positional value is provided by transplantation experiments. When a region of the body column is transplanted to a second animal, the implant will either form a head, form a foot, or be absorbed (e.g. Sugiyama, 1982). The simplest form of the experiment is to treat animals with the peptide, and then transplant a region of the body column to a similar location in a host (Fig. 4). Should the positional value be lowered, a larger fraction of the transplants will form feet (Sugiyama, 1982; Takano and Sugiyama, 1983).

Hydra magnipapillata were treated with a 10^{-6} M concentration of Hym-346 for 6 days or with a 10^{-8} M concentration for 15 days. Thereafter, a piece of the 2-region of treated animals was excised, and transplanted to the 2-region of untreated hosts. In both cases, the fraction of transplants forming feet increased signifcantly above the level found in untreated controls (Table 3).

In a variation of this experiment, the 3-region of treated animals was transplanted to the 1-region of the host. Although the fraction forming feet is invariably higher in this combination than in the previous one (Sugiyama, 1982; MacWilliams, 1983), donor tissue derived from peptide-treated animals formed an even higher fraction of secondary axes with feet than did tissue taken from the controls (Table 3). The results of these three experiments indicate that the peptide increased the probability of forming a foot in two different regions of the body column, which is consistent with a lowering of the positional value in the body column.

Finally, as a control to ensure that the increases in foot formation were due to changes in the transplant, the reciprocal experiment was carried out. The 2-region of control animals was transplanted into the 2-region of hosts treated with Hym-346. There was no difference in the fraction forming feet (Table 3) thereby providing evidence that the increases in foot formation observed in the other experiments were due to changes in the peptide-treated donor tissue.

Hym-346 increases foot formation in aggregates

Because the tissue of the body column of a hydra is always capable of forming either head or foot, it provides a useful substrate for testing the effects of molecules that might be involved in the patterning processes. An even better substrate are the tissues that develop during aggregate formation. When cells obtained by dissociating hydra into cell suspensions are

Table 3. Stimulation of foot formation in transplanted tissue treated with Hym-346

Experiment	Tissue	Treatment with Hym 346 concentration (M)	Length of time (days)	Source of donor tissue	Transplantation target site	Sample size (n)	Fraction forming feet (%)
1	Donor	10-6	6	2-region	2-region	128	12.5
	Donor	control	6	2-region	2-region	124	4.0
2	Donor	10-8	15	2-region	2-region	122	20.5
	Donor	control	15	2-region	2-region	102	11.8
3	Donor	10-8	15	3-region	1-region	96	90.9
	Donor	control	15	3-region	1-region	87	81.6
4	Host	10-6	6	2-region	2-region	92	7.6
	Host	control	6	2-region	2-region	90	8.9

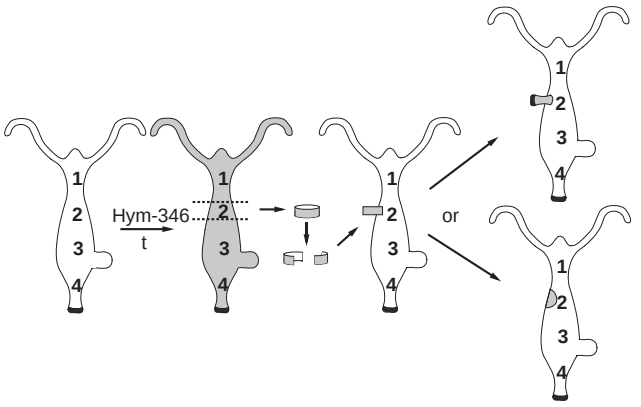

Fig. 4. Design of the transplantation experiment to examine the effect of Hym-346 on foot formation in *Hydra magnipapillata*.

centrifuged, the resulting pellet, or aggregate of cells, will develop into one or more hydra (Gierer et al., 1972). During this process, the positional value gradient and all other patterning processes must be re-established de novo. Thus, unlike an intact body column in which effects of a molecule are observed in terms of modifying the existing patterning processes, in a developing aggregate, the effects of the molecule are exerted as the patterning processes are being established. In principle, the effects of pedibin/Hym-346 on foot formation could be larger or more pronounced.

This possibility was tested by treating adults of the Basel strain of *Hydra vulgaris* with Hym-346 at 10^{-6} M for 15 days. Thereafter, the animals were dissociated into a suspension of cells, aggregates formed and treated with the peptide at the same concentration. Periodically, samples of developing aggregates were analyzed for the formation of heads and feet. Developing heads are easy to recognize morphologically during early stages of aggregate development as conical protrusions surrounded by two or more tentacles (Fig. 5). In contrast, a developing foot is difficult to recognize morphologically. Since *CnNK-2* is expressed in developing feet (Grens et al., 1996), spots or protrusions with *CnNK-2* are easily recognizable as developing feet in an aggregate and can be used as a marker for foot formation (Fig. 5). Expression of *CnNK-2* was analyzed by subjecting the aggregate samples to whole-mount in situ hybridization for this gene.

Normally heads begin to appear by 3 days after aggregation and feet a day later. This also occurred in the control aggregates in this experiment (Table 4). Usually, the foot:head ratio in an aggregate by 4-5 days is between 0.5 and 1, as was also found here. However, the numbers of head and feet formed in the aggregates made from peptide-treated animals were strikingly different. First, there was a sharp reduction (~8×) in the number of heads, and an even larger increase (45×) in the number of feet formed by day 3 (Table 4). This is evident by comparing the number of heads or feet per aggregate on day 3 for treated and control animals. This change is dramatically illustrated by a change in the foot: head ratio from 0.03 to 12.25. During the following 2 days, the number of heads increased to a level that was about half that of control levels. At the same time, the number of feet formed per aggregate was about 2× higher than normal. Consequently, the foot:head ratio remained 2-3× higher in peptide-treated aggregates compared to controls.

In a similar experiment animals of the L2 strain of *H. vulgaris* were treated with different concentrations (10^{-12}-10^{-6} M) of pedibin for 7 days, aggregates formed, and were analyzed for head and foot formation as described above. Although the differences were less pronounced, the same tendency was observed in that there was an increased level of foot formation with increasing concentrations of pedibin (data not shown).

Thus, these results indicate that treatment with pedibin/Hym-346 significantly alters the patterning processes in a developing aggregate in favor of foot formation. Feet are formed earlier, more are formed and, conversely, fewer heads are formed.

DISCUSSION

The positional value gradient maintains the form of an adult hydra in the context of its tissue dynamics (e.g. Wolpert et al., 1971). The basis of positional value has been described both in terms of a pair of gradients, one each for the head and foot with maxima in the respective extremity (e.g. Bode and Bode, 1984a), or more recently as a single gradient (e.g. Müller, 1996). Since the axial location of a given piece of tissue of the body column is continuously changing, the positional value of that piece of tissue must also be continuously changing. For example, as the tissue of the upper part of the column is displaced apically towards the head, its positional value rises. This implies that the gradient is dynamic, not static, and that most likely there are signal(s) that act continuously to maintain the gradient. Since the body column is 80-100 cell diameters in length, the signal(s) is probably operating over a long distance.

The molecular nature of the positional value gradient is poorly understood. The ability to raise and lower the positional value along the body column with diacylglycerol (Müller, 1990) and LiCl (Maggiore and H. R. B., unpublished data),

Table 4. Effect of Hym-346 on head and foot formation in aggregates

Aggregate age (days)	Hym-346 treatment	Sample size (*n*)	Structures/aggregate Heads	Feet	Foot/Head
3	–	25	1.24±0.44	0.04±0.20	0.03
4	–	25	2.16±0.94	1.72±0.68	0.80
5	–	24	4.13±1.13	1.66±0.70	0.40
3	+	27	0.15±0.36	1.81±0.56	12.25
4	+	27	1.47±0.66	3.07±1.00	2.09
5	+	25	2.08±0.87	3.00±1.15	1.44

Error is the standard deviation.

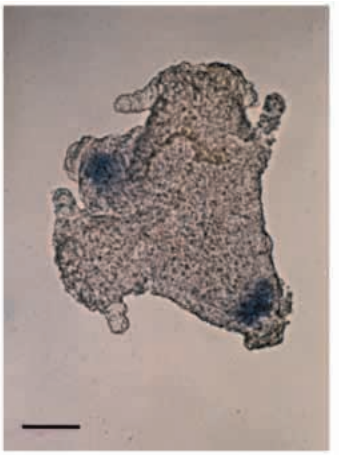

Fig. 5. Development of head and foot structures in 5 day control aggregates of cells of *Hydra vulgaris*. Each of the two developing heads is recognizable as a group of two or more tentacles surrounding a hypostome, while the two developing feet are identified by the expression of *CnNK-2*. Scale bar represents 0.4 mm.

respectively, suggests an involvement of the phosphatidylinositol cycle. Although evidence has been obtained previously that three different peptides affect the rates of head and foot regeneration (Schaller, 1973; Hoffmeister, 1996), the results described here are the first systematic effort to determine if a peptide affects the positional value gradient and, thus, may be involved in the patterning process.

Here we have shown that two closely related peptides, pedibin of *Hydra vulgaris* (Hoffmeister, 1996) and Hym-346 of *Hydra magnipapillata* (Takahashi et al., 1997), have similar biological activities. Since both have similar effects on the positional value gradient and foot regeneration, it is likely that they play the same role in patterning processes in hydra. Thus, the additional glutamine at the C-terminal end of Hym-346, which represents the one difference in the amino acid sequence of the two peptides, probably represents a species difference that is not important for the activity of the peptide.

Effect of pedibin /Hym-346 on positional value

Several experiments lead to the view that the effect of pedibin/Hym-346 is to increase foot-forming potential, or phrased differently, to lower the positional value.

(1) *CnNK-2* is expressed in a graded distribution up the body column (Grens et al., 1996). Lowering of the positinal value gradient leads to an apical extension of the range of expression of this gene. Treatment with pedibin/Hym-346 causes the same change in the expression of *CnNK-2*.

(2) In transplantation experiments, the donor tissue will form either a head or foot or will be absorbed. Transplants from peptide-treated animals formed feet more frequently than did controls.

(3) The aggregation experiments provide an even more sensitive measure of the positional value of the tissue. In this situation, the pattern in the tissue has been destroyed and must be set up de novo. However, the cells retain a memory of their positional value. In aggregates made from isolated body columns, head and feet are formed in normal proportions (Gierer et al., 1972). In contrast, if the aggregates are made from head tissue alone, large numbers of heads, but few feet, are formed (Gierer et al., 1972; Schaller, 1975). When the isolated body columns of animals treated with Hym-346 were dissociated and aggregates formed, the resulting aggregates formed more feet than heads.

Hence, the results of these three types of experiments indicate that pedibin/Hym-346 lowers the positional value of the tissue and, hence, increases the probability of the tissue forming a foot. What is the explicit role of pedibin/Hym-346? One possibility is that it is a signal that commits tissue to foot formation. The argument for a role in foot formation is the following.

In an adult hydra, commitment of tissue to foot formation is an event that occurs continuously. As part of the tissue movements, tissue is continuously displaced down the column through the peduncle and then into the foot (Campbell, 1967). Once tissue reaches the lower peduncle, the region at the base of the body column just above the foot, it is committed to foot formation (Bode and Bode, 1984b). *CnNK-2* expression provides a marker for this commitment. Not only is this gene expressed at a high level in the lower peduncle, but a high level of *CnNK-2* expression always coincides with commitment of tissue to foot formation in a variety of experimental manipulations (Grens et al., 1996). Further, the graded expression of *CnNK-2* in the adult is due to a signal emitted by the lower peduncle (Grens et al., 1996). Taken together, these facts suggest that the lower peduncle emits a signal that is involved in committing tissue to foot formation. Since pedibin/Hym-346 not only causes an apical extension of the range of CnNK-2 expression but also increases the probability of foot formation in the transplantation and aggregation experiments, this peptide could be that signal.

However, the situation is probably a step more complicated. The stored form, or source, of pedibin, and thus most likely also of Hym-346, is known to be fairly uniformly distributed along the body column (Hoffmeister, 1996) instead of being localized only in the peduncle. This suggests a mechanism that results in the preferential release of pedibin/Hym-346 at the lower end of the column where it is subsequently active.

Effect of pedibin/Hym-346 on foot regeneration

As shown previously by Hoffmeister (1996) for pedibin, and shown here for Hym-346, both peptides increase the rate of foot regeneration following bisection of the body column, or after isolation of the peduncle. In all three experiments, treatment with a low concentration of peptide (10^{-10}-10^{-9} M) for a short period of time (1 day before, or 1 day after bisection, or both) was sufficient to detect a significant increase in the rate of foot regeneration. In contrast, the observed effects on lowering the positional value in the intact animal required longer exposures (6-15 days) with higher concentrations (10^{-8}-10^{-6} M). This raises the question whether pedibin/Hym-346 is affecting the same or two different processes for foot regeneration and the lowering of positional value. Although at first glance, the two effects appear to be different, the accumulated evidence on head regeneration provides an argument suggesting that the pedibin/Hym-346 is acting in a similar manner in both situations.

Upon bisection of the body column, a head regenerates at the apical end of the lower part of the animal. As measured in transplantation studies (MacWilliams, 1983), the tissue at the

regenerating tip is rapidly committed to head formation (T_{50}=8 hours when bisection is directly below the head). Another measure is the expression of genes specifically expressed in the head of hydra such as *Budhead* (Martinez et al., 1997) and *Hybra-1* (U. Technau and H. R. B., unpublished results), which begins soon (3-8 hours) after decapitation. The head activator, an 11-amino acid peptide is known to play a role in this process as it increases the rate of head regeneration when added to decapitated animals (Schaller, 1973; Javois and Tombe, 1991). Further, bisection results in the release of stored head activator into the surrounding environment (Schaller et al., 1986). This release of head activator could be part of the cascade of events resulting in commitment of the tissue to head formation and subsequent head regeneration. Addition of external head activator would increase the concentration of head activator and simply speed up the events leading to commitment and/or head regeneration.

Pedibin/Hym-346 could play a similar role for foot formation. Since pedibin is known to be stored (Hoffmeister, 1996), injury could cause the release of the peptide which then has a role in foot formation. Addition of external pedibin/Hym-346 would then increase the rate of commitment and subsequent rate of foot regeneration. The fact that there is five times less stored pedibin in a strain of *Hydra oligactis* (Hoffmeister, 1996), which has a reduced capacity for foot regeneration (Hoffmeister, 1991), provides further support for this idea.

If pedibin/Hym-346 has a similar role in foot regeneration, and in alteration of the positional value, why are different concentrations of the peptide and different lengths of exposure required to observe increases in foot formation in the two processes? The processes differ in two respects.

(1) In the regeneration experiments, the cells and tissue involved in regeneration are directly exposed to the peptide and, thus, the low concentrations are probably an accurate reflection of the required concentration to increase the rate of regeneration. In contrast, in treating intact animals to alter the positional value, the peptide must enter the intercellular spaces in the tissue through the septate junctions connecting the apical ends of the lateral edges of the ectodermal epithelial cells. Hence, the actual concentration of peptide that the target cells are exposed to is probably much lower than the concentration in the surrounding medium in which the animals are bathed and may be closer to that found to be effective in the regeneration experiments.

(2) The second aspect concerns the time of exposure. During foot regeneration, the peptide acts synergistically with the ongoing regeneration process thereby increasing the rate of regeneration. In contrast, changes in positional value may take longer. Because the patterning processes to maintain the positional value gradient are constantly active, and the introduced peptide would be acting counter to part of this process, the changes would be much slower.

In sum, the simplest explanation is that pedibin/Hym-346 has a role in the process governing commitment to foot formation in the body column in hydra. This process is an integral part of maintaining the positional value gradient along the column and that the changes observed in the regeneration, transplantation and aggregation experiments, as well as the expression patterns of *CnNK-2,* are all manifestations of this role. Current efforts to isolate the gene encoding Hym-346, as well as to determine in which cell types it is expressed, will further elucidate the role of pedibin/Hym-346.

Peptides and pattern formation

Currently the signaling molecules known to affect pattern formation in hydra and other cnidarians are peptides, while in more complex animals such signaling molecules have been identified as proteins. This could reflect a fundamental difference between the cnidaria, which arose early in metazoan evolution, and other taxa, which appeared later. Or, it could simply reflect the focus of efforts so far in the different groups of organisms. That proteins known to affect developmental processes in more complex organisms also play a role in hydra has been demonstrated by Yan et al. (1995). They have shown that FGF and activin, which affect patterning in vertebrates, stimulate cell proliferation in hydra. Effects on patterning processes were not examined. Conversely, analysis of the role of peptides such as pedibin/Hym-346 may show that they play a role in patterning processes, or other developmental processes, in more complex organisms.

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