The novel peptide HEADY specifies apical fate in a simple radially symmetric metazoan

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One of the first steps in animal development is axis formation, during which an uneven distribution of signals and/or transcription factors results in the establishment of polarity in the embryo. *Hydra*, one of the simplest metazoan animals, shows characteristics of a permanent embryo. Even adult polyps have a striking capacity to regenerate, suggesting that molecular mechanisms underlying de novo pattern formation are permanently active and self regulatory. Here we show that HEADY, a short, amidated peptide, plays a central role in the specification of apical fate in this simple metazoan. The *HEADY* gene, whose transcripts accumulate at the apical organizing center, is required for specification of apical fate, as disruption of *HEADY* function by dsRNA mediated interference (RNAi) results in severe defects in head formation. Conversely, an instructive role of HEADY in head specification is demonstrated by the application of synthetic HEADY peptide, which induces formation of secondary axes with head morphology. Thus, the HEADY peptide acts as developmental switch to pattern the apical-basal axis of *Hydra*, providing a first insight into how initial asymmetry is specified in lower metazoan animals.

[Key Words: *Hydra*; peptides; head induction; regeneration; DD-PCR; RNAi]

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Hydra is a solitary freshwater polyp and a member of the basal phylum of cnidaria. Animals from this phylum were the first in metazoan evolution to develop a complex body structure composed of specialized tissues. *Hydra* polyps are radially symmetric and, therefore, have only one prominent axis, the apical-basal body axis. Head and foot are the only differentiated structures of the animal and define the opposite ends of this axis (Bosch 1998). Polyps proliferate asexually by budding, a process during which a secondary polyp forms in the lower gastric region of the parental animal. *Hydra* has a diploblastic body plan and consists of only two cell layers. Cell proliferation takes place continuously in both of the cell layers but is restricted to the gastric region of the animal. Mitotically produced cells are displaced toward head and foot, where they differentiate to form the appropriate structures (David and Campbell 1972). Cells are continuously lost from the adhesive foot and the tentacles, which are used to catch prey. Therefore, tissue dynamics have to be tightly controlled to maintain homeostasis between the two cell layers as well as between the different cell types within the layers. To maintain this steady state of proliferation and differentiation, un-

derlying developmental signals have to be continuously active to provide cells with positional information throughout the entire life cycle of the animal (Bosch 1998). The molecular nature of these signals is still largely unknown, but there is good evidence that patterning in *Hydra* is governed by self-regulatory interacting gradients of diffusible factors (Meinhardt 1993). It is thought that independent reaction-diffusion systems regulate the development of the head and foot, as well as the formation of buds. The continuous presence of these developmental signals gives even adult polyps a remarkable ability to fully regenerate lost body structures. These observations suggest that in *Hydra* axis formation, fate specification and differentiation are inseparably linked and continuously active processes. In this study, we demonstrate that in addition to such continuously active processes, HEADY, a newly discovered signaling peptide, acts transiently to specify the apical end of the body axis.

Results

Isolation and sequence analysis of the HEADY cDNA

To identify genes involved in axis formation and patterning in simple metazoans, we developed a screening strategy that makes use of the remarkable regeneration capacity of the freshwater polyp *Hydra*. Intact polyps were dissociated into single cells and kept in suspension for 4 h before they were reaggregated by centrifugation (Gierer et al. 1972). By de novo axis formation, these cell aggre-

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Figure 1. HEADY mRNA and protein structure. (A) Structure of the HEADY transcript. Nucleotides 54–122 encode the HEADY precursor protein. The putative leader sequence is shown in blue, processing site in black, and the propeptide in green. The complete HEADY cDNA sequence has been deposited in GenBank under accession AF188478. (B) cDNA and deduced amino acid sequence of the HEADY precursor. The ATG triplet in position 54 is the first start codon in the transcript. The G in the fourth position indicates that it is a preferred site for initiation of translation (Kozak 1997). The deduced amino acid sequence of the HEADY precursor is shown below the cDNA sequence. (C) Sequence of the mature HEADY peptide.

gates regenerate into intact polyps within a few days (Gierer et al. 1972). RNA samples were taken from intact polyps, from cells 2 min after dissociation, from cells 4 h after dissociation, from cells 1.5 h after reaggregation, and from cells 20 h after reaggregation. These RNA

samples were then subjected to nonradioactive differential display PCR (Liang and Pardee 1992; Lohmann et al. 1995) to monitor gene expression during de novo axis formation. Fourteen of 450 transcripts (3%) analyzed in two independent experiments were found to be differentially expressed. One of these was represented in a short cDNA of ~380 bases predicted to encode a short peptide that we named HEADY (Fig. 1). Sequence analysis revealed an open reading frame of 69 bases coding for the peptide precursor. By analogy with other cnidarian peptides (Darmer et al. 1998), the propeptide could be cleaved from the precursor after the motif arginine-asparagine. As a C-terminal glycine-aspartate motif was recently found to serve as amidation signal in the cnidarian peptide Hym-357 (Yum et al. 1998a), the mature Hedy peptide is predicted to have the structure FHTMILLDTQSP-NH₂ (Fig. 1C). To confirm the structure-function relationship of the HEADY peptide, we investigated the biological activity of synthetic full-length and amidated peptide FHTMILLDTQSP-NH₂, the N-terminal deleted version TQSP-NH₂, and the two nonamidated peptides FHTMILLDTQSPGD and FHTMILLDTQSPG (see Materials and Methods). Only the full-length and amidated peptide showed significant biological effects, confirming the predicted peptide structure.

Expression of HEADY is transient and specific to apical organizing centers

HEADY RNA expression is spatially and temporally regulated (Fig. 2). Using RT-PCR, we found that HEADY, which was only weakly expressed in intact pol-

Figure 2. Expression of HEADY is transiently induced during apical fate specification. (A) Expression of HEADY during head regeneration. RT-PCR revealed a maximum of HEADY expression 6 h after onset of head regeneration (R6). After 8 h (R8) expression was again reduced considerably. Actin was used as control for equal loading of cDNA; minus sign indicates water control. (B) Whole-mount in situ hybridization of a head-regenerating animal 6 h after decapitation. (C) Cells expressing HEADY were located in the endoderm at the apical-most position of the regenerating tip (30 min of color reaction), and (D) 90 min of color reaction revealed that the level of HEADY mRNA decreases in a gradient from apical to more basal tissue. (E) Intact polyp, no staining could be detected. (F–I) Expression of HEADY during budding. (F) HEADY expression could be detected first in bud stage 3 (Otto and Campbell 1977). (G) During intermediate budding stages (stage 5), HEADY transcripts were restricted to the endoderm with increased amounts of mRNA at the apical tip of the bud. (H) During later stages of budding, with tentacles starting to be formed, HEADY expression was strong and restricted to the apical-most cells of the endoderm. (I) In a mature bud (bud stage 9) that will detach from the parental polyp within the next few hours, HEADY transcripts were located in the apical most endoderm only. No transcripts were found in the tentacles.

yps, is transiently up-regulated 6 h after decapitation (Fig. 2A). The induction was similar regardless of whether animals were bisected just below the tentacles or in the midgastric region. This was surprising because the potential for head regeneration decreases from apical to basal positions (Bosch 1998), leading to a delayed formation of head structures from basal tissue. Correspondingly, the induction of genes linked to head differentiation in *Hydra* is delayed when animals are decapitated in more basal positions (Endl et al. 1999; Technau and Bode 1999). These results suggest that the HEADY peptide is not involved in head differentiation, which is position dependent, but rather in the preceding fate specification. No increase in *HEADY* expression could be observed when the foot was removed, confirming that the induction was specific for apical determination and was not caused by simple wounding. In situ hybridization (Martinez et al. 1997) showed that during apical fate specification *HEADY* transcripts accumulate in a small number of endodermal cells at the regenerating tip, with highest levels in the apical-most cells (Fig. 2B–D). In intact animals, no localized accumulation of *HEADY* transcripts could be detected (Fig. 2E).

To confirm that HEADY activity participates in the general mechanism to specify the apical end of the body axis and is not restricted to head regeneration, we analyzed *HEADY* expression during budding. During this process, a secondary axis is induced without injury. From bud stage 3 (Otto and Campbell 1977) on, *HEADY* transcripts accumulated evenly throughout the endoderm of the bud (Fig. 2F). During subsequent development of the bud, expression was progressively confined to the apical end (Fig. 2G–I). A few hours after the bud detached from the parental animal, *HEADY* transcripts were no longer present in the head of the young polyp (Fig. 2E). Thus, early apical fate specification and *HEADY* expression are intimately linked.

HEADY peptide is stored in vesicles in endodermal epithelial cells

To localize the mature HEADY peptide in *Hydra* tissue, a polyclonal anti-HEADY antiserum was raised in chicken. In competitive ELISA, a 1 : 8000 dilution of the antiserum was shown to bind to the HEADY peptide even in the presence of 10 $\times$ molar excess of unrelated protein (data not shown), indicating specific affinity to the HEADY peptide. Using this antiserum for whole-mount immunocytochemistry, we detected HEADY peptide in vesicles of endodermal epithelial cells, evenly distributed throughout the gastric tissue but almost absent in head or foot (Fig. 3C). HEADY seemed to be released in early stages of bud formation, as stained vesicles could not be detected in this tissue (Fig. 3D). Similarly, in head regeneration experiments, no staining was seen in the regenerating tip immediately following decapitation (data not shown). Staining was reestablished 8 h after the onset of regeneration, indicating a transient and local release of HEADY peptide in tissue undergoing apical fate specification. Depletion of

Figure 3. Immunolocalization of mature HEADY peptide in vesicles of endodermal epithelial cells in the gastric region. Confocal micrographs of whole-mount immunocytochemistry are shown. Scale bars indicate 30 μ m. (A) Representative pictures (B,C,D) were taken at the sites indicated by squares. The confocal plane was set to the endoderm. (B) Midgastric tissue stained with preimmune serum. No specific immunofluorescence could be detected. (C) Midgastric tissue stained with anti-HEADY serum. Fluorescence was observed in endodermal epithelial cells. Higher magnification (insert in C, scale bar = 1 μ m) revealed that each spot of fluorescence consists of several HEADY-containing vesicles. About 20% of the endodermal epithelial cell population was found to contain HEADY-positive vesicles. (D) Tip of a young bud (stage 3) stained with anti-HEADY serum. Absence of HEADY-containing vesicles suggested that it had been released in the course of early bud formation. Staining at the edge of the tissue sample could also be detected with the preimmune serum and was regarded to be unspecific.

HEADY-containing vesicles was followed by up-regulation of *HEADY* transcription as revealed by in situ hybridization and RT-PCR (see Fig. 2). Strikingly, although expression was up-regulated during the early specification of the apical axis end, neither *HEADY* transcripts nor the mature peptide were differentially localized in the adult head (Fig. 2). This supports the view that HEADY plays a role only in the induction of the head, not in its maintenance.

HEADY induction is absent in a regeneration-deficient mutant

To further explore the correlation of head induction and *HEADY* activity, we studied *HEADY* expression in the *reg16* mutant, whose potential for head regeneration is strongly reduced (Sugiyama and Fujisawa 1977). In intact *reg16* polyps, *HEADY* levels were not changed compared

to the wild-type strain. In contrast to wild-type, however, *HEADY* expression was not induced in decapitated *reg16* polyps. When the regeneration capacity of *reg16* was restored by repeated wounding (Achermann and Sugiyama 1985) or treatment with the PKC activator TPA (Weinziger et al. 1994; Müller 1995), *HEADY* expression was restored to wild-type levels (data not shown), confirming a specific correlation between head induction and *HEADY* expression.

HEADY is necessary for head induction

To determine whether *HEADY* was required for head induction in wild-type polyps, we generated *heady* loss-of-function animals by dsRNA-mediated interference (Fire et al. 1998). *HEADY* dsRNA was synthesized and electroporated into intact polyps according to previously established protocols (Lohmann et al. 1999). After 4 d, polyps showed drastic depletion of *HEADY* transcripts (Fig. 4B). When those *HEADY* loss-of-function animals were decapitated, their capacity to regenerate a new head was severely reduced compared with mock-electroporated animals (Fig. 4A,C). On average, head formation was delayed by about 2 d but was not completely blocked. This is compatible with *HEADY* expression being only reduced, not eliminated, by RNAi. Because of the continuous cell proliferation in *Hydra*, the amount of dsRNA per cell is increasingly diluted. Thus, the gene-silencing effect is progressively decreased, allowing *HEADY* transcripts to accumulate with time. Therefore, residual *HEADY* levels of as little as 10% may be sufficient to promote head regeneration, although at a slower rate. Alternatively, head induction might be redundantly regulated, with other factors partly substituting for *HEADY* activity.

No effect of *heady* loss of function was observed in foot-regenerating animals (data not shown), indicating an essential role for *HEADY* in the specification of the apical but not the basal end of the body axis.

HEADY peptide is an inducer of apical fate

Having demonstrated that *HEADY* is necessary for the specification of apical fate and, thus, head formation, we tested whether the *HEADY* peptide is also sufficient for head induction. Because a secondary head is induced during budding, we first studied the influence of synthetic *HEADY* peptide on this process. Newly detached polyps were continuously incubated for 12 d in 10^{-6} M *HEADY* peptide, fed daily, and scored for number of buds. Exogenously applied *HEADY* caused budding to be initiated ~1.5 d earlier compared to controls (Fig. 5A). Once the animals started to bud, however, the number of buds produced per day (budding rate) was not increased. As there was no influence of *Heady* on the rate of budding, the peptide does not appear to affect cell proliferation but, rather, to induce precocious secondary head formation. To verify this hypothesis, we tested the potential of *HEADY* to induce apical fate in lateral grafting experi-

Figure 4. RNAi with *HEADY* disrupts head regeneration. (A) Schematic outline of the RNAi procedure (Lohmann et al. 1999). (B) Depletion of *HEADY* mRNA by RNAi as shown by RT-PCR using *HEADY*-specific primers. Actin was included as loading control. (C) RNAi of *HEADY* dsRNA reduced the rate of head regeneration 30 h after decapitation from 10% ($n = 99$) in mock electroporated control animals to 1.3% ($n = 114$) in *HEADY* dsRNA electroporated animals. A total of 240 young polyps were electroporated with 20 μ g of *HEADY* dsRNA or were mock electroporated. After 4 d of recovery, animals were decapitated and allowed to regenerate for 30 h. Only fully regenerated heads (Lohmann et al. 1999) were scored.

ments. Using this assay, the morphogenetic activity of tissue can be tested without interference from cell proliferation effects. Polyps were continuously incubated in 10^{-6} M peptide with daily feeding for 5 d and then used as donors for homotopic gastric transplantation. Small pieces of donor gastric tissue were transplanted into the same gastric position in untreated host polyps (Fig. 5B; Sugiyama 1982). Secondary axes with head morphology formed 30% of *HEADY*-treated implants, compared with only 10% in controls. Foot formation was markedly reduced in tissue treated with *HEADY* before transplantation, confirming that *HEADY* is a potent and specific inducer of apical fate.

HEADY is not involved in maintaining the pattern along the apical-basal axis

To elucidate whether *HEADY* also plays a role in continuous head formation in intact polyps, we examined

Figure 5. HEADY peptide affects head induction in *Hydra*. (A) Bud formation in HEADY-treated polyps occurred 1.5 d earlier than in untreated control animals ($n = 60$ polyps). The rate of budding, however, was not affected. (B) Experimental outline of grafting procedure (Sugiyama 1982). (C) Control implant (arrowhead) showing no differentiated structures. (D) HEADY-treated implant (arrowhead) developing a secondary axis with head morphology. B, developing bud on the primary body axis. (E) Stimulation of secondary axis formation in transplanted tissue treated with HEADY. Five days after transplantation 30% ($n = 79$) of the treated implants showed developed heads (see D), compared with 10% ($n = 75$) in untreated control implants (C). Foot formation was induced in 20% of control implants and was reduced to 13% in HEADY treated tissue.

the expression of several developmental regulators in reaction to HEADY peptide treatment. Animals were incubated in 10^{-6} M HEADY for 7 d, followed by expression-analysis of the transcription factor genes *Budhead* (Martinez et al. 1997) and *CnNK-2* (Grens et al. 1996), as well as the matrix metalloprotease gene *FARM1* (Kumpfmüller et al. 1999) by in situ hybridization or RT-PCR. These genes have been shown to be sensitive to head-, foot-, or gastric-specific signals and appear to play essential roles in maintaining the pattern along the *Hydra* body axis (Grens et al. 1996, 1999; Martinez et al. 1997; Kumpfmüller et al. 1999). HEADY treatment did not influence their expression (data not shown), supporting the view that HEADY is involved in apical fate specification but does not act as a positional signal once the pattern along the apical-basal axis has been established.

Discussion

A screen for transcripts induced during de novo axis formation and morphogenesis in the basal metazoan *Hydra* resulted in the isolation of *HEADY*, an RNA coding for a short, amidated peptide. In this study, we demonstrated that the activity of the HEADY peptide is both necessary and sufficient to organize the apical end of the body axis in *Hydra*.

HEADY cDNA and protein structure

The *HEADY* transcript has several unusual features. It is the shortest mRNA isolated from Cnidarians to date. Other cDNAs coding for peptide precursors isolated from *Hydra* so far range in size from 600 bp to 1.5 kb (Darmer et al. 1998; Yum et al. 1998a). The *HEADY* cDNA exhibits a short open reading frame of 69 bases that codes for a single peptide. Previously described peptide precursors contain two to 11 individual peptides

(Darmer et al. 1998; Gajewski et al. 1998). Because of the small size of the HEADY precursor, it seems unlikely that the nascent protein is translocated to the endoplasmic reticulum (ER) during translation. Rather, it seems that the 23-amino acid precursor is translocated to the ER posttranslationally in a process similar to the one employed by the yeast α factor (Hansen et al. 1986). Although the native HEADY peptide has not been isolated from *Hydra* tissue so far, a number of experimental observations confirm that the isolated cDNA codes for the predicted active signaling molecule. First, depletion of *HEADY* transcripts by RNAi (loss of function) and the application of HEADY peptide to polyps (gain of function) result in opposite effects. Second, only the synthetic peptide of the predicted structure exhibits biological activity. Third, release of HEADY peptide from vesicles is associated with colocalized up-regulation of *HEADY* transcription.

HEADY, a potent, transient inducer of apical fate

The continuous proliferation and differentiation of cells in *Hydra* suggests that underlying developmental programs are continuously active. This is reflected in theoretical models based on reaction-diffusion equations that are capable to simulate all aspects of *Hydra* development (Meinhardt 1993). These models predict self-regulatory morphogen gradients that are continuously present and active throughout the entire life cycle of the polyp. Classical transplantation experiments have revealed a number of morphogenetic activities in *Hydra* tissue, including head or foot induction or inhibition (MacWilliams 1983a,b). However, the underlying molecules have not been identified to date. The isolation and characterization of HEADY reveals that the regulatory network of head formation in simple metazoans is more complex than anticipated. The fact that *HEADY* expression does not follow the gradient of head activation capacity and

the finding of local and transient release of the peptide demonstrates that HEADY is involved in the specification of apical fate rather than in the process of head differentiation. This is in contrast to all other genes that have been reported to be linked to head formation in *Hydra* (Shenk et al. 1993; Martinez et al. 1997; Endl et al. 1999; Technau and Bode 1999) and provides first evidence that apical fate determination and head formation are two closely linked but independent processes. Another candidate for one of the signals involved in head development is the neuropeptide head activator (HA; Schaller 1973). Although HEADY shares no sequence similarity with the HA, its effect on bud formation seems to be similar (Hobmayer et al. 1997). However, unlike HA, HEADY treatment does not increase the number of tentacles during head regeneration, suggesting that HEADY and HA act in distinct genetic pathways. Furthermore, HA is localized in the mature head (Hampe et al. 1999), where HEADY peptide is virtually absent.

Peptides, key molecules in metazoan evolution

A growing body of evidence indicates that peptides play important roles as developmental signals in lower metazoans. First, a large number of peptide signaling molecules have been isolated from *Hydra* and other Cnidarians by various experimental approaches (Bodenmüller and Schaller 1981; Leitz et al. 1994; Hoffmeister 1996; Takahashi et al. 1997, 2000). Recent experiments suggest that *Hydra* contains about 1000 distinct peptide molecules (T. Takahashi, T. Fujisawa, pers. comm.). In contrast, no molecule related to growth factors of higher animals has been isolated to date. Second, peptides serve in a variety of biological processes as diverse as metamorphosis induction (Leitz et al. 1994), muscle contraction (Yum et al. 1998b), nerve cell differentiation (Takahashi et al. 2000), or apical fate specification and, therefore, are not limited to certain aspects of development. Third, their small size makes peptides ideal molecules for traveling with ease in the interepithelial space to cause the long-range effects necessary for the establishment of morphogenetic gradients. Our data on the isolation and functional analysis of HEADY adds further support to the view that peptides have played a key role as developmental signals in metazoan evolution.

Materials and methods

Animals

Hydra magnipapillata polyps were cultured according to standard procedures at 18°C.

PCR

HEADY cDNA was isolated from a DD-PCR reaction using poly(A)⁺ RNA from regenerating cell-aggregates and primers T₁₂AG and AGTCAGCCAC. Primers 5'-AGTCAGCCACAGCAACATAGG-3' and 5'-AGTCAGCCACTCGGGGAGC-3'

were used for specific quantitative RT-PCR. cDNA pools of 20 polyps were used as template for amplification of each time point. RACE PCR (Chenchik et al. 1996) was used to isolate the 369-bp HEADY cDNA.

Antisera

The anti-HEADY polyclonal antiserum was raised in chicken against KLH-coupled TQSP-NH₂. FITC-conjugated antichick Fab fragments were used as a secondary antibody.

Peptide treatments

Peptide stock solutions were kept as 10⁻³ M at -80°C. Working solutions were prepared freshly for each experiment by diluting the stock to 10⁻⁶ M with *Hydra* culture solution. To determine the structure of the mature and biologically active HEADY peptide, we tested peptides FHTMILLDTQSP-NH₂ (predicted structure), TQSP-NH₂, FHTMILLDTQSPGD, and FHTMILLDTQSPG in the budding assay as shown in Figure 5A. As only the predicted peptide was capable of influencing budding, we concluded that FHTMILLDTQSP-NH₂ is the active form.

TPA treatment

Polyps were incubated in 30 nM TPA (12-O-Tetradecanoylphorbol-13-acetat; Sigma) in *Hydra* culture solution for 20 min as described (Weinziger et al. 1994) and washed twice in culture solution immediately. After another 30 min in culture solution, experiments were carried out.

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