Silencing of the hydra serine protease inhibitor Kazal1 gene mimics the human SPINK1 pancreatic phenotype

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Abstract

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Silencing of the hydra serine protease inhibitor Kazal1 gene mimics the human SPINK1 pancreatic phenotype

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Summary

In hydra, the endodermal epithelial cells carry out the digestive function together with the gland cells that produce zymogens and express the evolutionarily conserved gene Kazal1. To assess the hydra Kazal1 function, we silenced gene expression through double-stranded RNA feeding. A progressive Kazal1 silencing affected homeostatic conditions as evidenced by the low budding rate and the induced animal death. Concomitantly, a dramatic disorganization followed by a massive death of gland cells was observed, whereas the cytoplasm of digestive cells became highly vacuolated. The presence of mitochondria and late endosomes within those vacuoles assigned them as autophagosomes. The enhanced Kazal1 expression in regenerating tips was strongly diminished in Kazal1(−) hydra, and the amputation stress led to an immediate disorganization of the gland cells, vacuolization of the digestive cells and death after prolonged silencing. This first cellular phenotype resulting from a gene knockdown in cnidarians suggests that the Kazal1 serine-protease-inhibitor activity is required to prevent excessive autophagy in intact hydra and to exert a cytoprotective function to survive the amputation stress. Interestingly, these functions parallel the pancreatic autophagy phenotype observed upon mutation within the Kazal domain of the SPINK1 and SPINK3 genes in human and mice, respectively.

Supplementary material available online at http://jcs.biologists.org/cgi/content/full/119/05/846/DC1

Key words: Kazal domain, SPINK1/SPINK3 serine protease inhibitor, Autophagy, Autophagosome, RNA interference, Evolution

Introduction

Owing to its position as sister group to bilaterians, Cnidaria provides us with crucial information on bilaterian history. Cnidarians, although lacking true organs, share with bilaterians a tissue organization, an embryonic development that traverses gastrulation (Bouillon, 1994), a behaviour controlled by a neuromuscular system (Westfall, 1996; Seipel and Schmid, 2005) and a large number of evolutionarily-conserved genes similarly involved in cell differentiation and/or developmental tasks (Galliot, 2000; Steele, 2002; Kusserow et al., 2005). Hydra is organized along a single oral-aboral axis that is divided into three parts: the foot region, the body column corresponding to the gastric cavity, and the apical head region with the mouth opening surrounded by a ring of tentacles. The two cell layers, ectoderm and endoderm, separated by the extracellular mesoglea, comprise epithelial cells and interstitial cells, from which neurons, nematocytes (stinging cells) and gland cells derive. Gland cells produce zymogens that are released in the gastric cavity and as such resemble pancreatic cells in vertebrates (Lentz, 1966).

Metalloproteinases are highly conserved multifunctional enzymes, involved in morphogenetic as well as digestive processes in cnidarians (Pan et al., 1998; Sarras et al., 2002). In bilaterians, protease inhibitors restrict their activity according to a tight balance that prevents cellular defects as autodigestion (Witt et al., 2000), abnormal cell migration and metastasis. Related protease inhibitors were identified in cnidarians (Tschescche et al., 1987; Holstein et al., 1992; Yang et al., 2003). Amongst those, the sea anemone elastase inhibitor and the hydra antistatin act as serine protease inhibitors, the former one encoding a Kazal domain (KD), initially characterized in pancreatic tissue as trypsin inhibitor (Rawlings et al., 2004). KDS are 40 to 60 amino acids (aa) long and contain six cystein residues that form disulfide bridges; their protease inhibitor activity was documented in vertebrates (Kazal et al., 1948), blood-sucking insects (Mende et al., 1999), animal parasites as inhibitors of host digestive enzymes (Morris et al., 2002) and plant parasites (Tian et al., 2004). We isolated the hydra gene Kazal1, which encodes three KDS and exhibits a high level of expression in gland cells. To see whether such a protease inhibitor in cnidarians elicits an effect related to those known in vertebrates, we tested this gene for its implication in protease inhibition by using double-stranded RNA interference (dsRNAi).

dsRNAi has proved to be a powerful tool, both to look at gene function and to implement systematic functional screens in contexts not amenable to other genetic approaches (Novina and Sharp, 2004). It was applied to hydra using electroporation as a delivery route (Lohmann et al., 1999). Although this method led to major side effects as tissue necrosis and lethality, it highlighted the function of several genes in head regeneration (Lohmann and Bosch, 2000; Smith et al., 2000; Cardenas and...
Salgado, 2003). However, patterning events such as maintenance, budding and sexual development, take place continuously in hydra and cannot be induced at a precise time. Therefore, we developed a milder strategy for dsRNAi that allows repeated treatments of Hydralus vulgaris (Hv) and H. magnipapillata (Hm) through feeding, a strategy that provides a stepwise and efficient gene-silencing effect. The delivery of dsRNAs by feeding the animal on dsRNA-producing bacteria was originally described in C. elegans (Timmons et al., 2001). As hydra does not spontaneously feed on bacteria, we adapted the solution set up in planarians, where bacteria are embedded into agarose as part of an artificial food mix (Newmark et al., 2003). We report here, that knocking down the hydra Kazal1 gene induces a phenotype surprisingly related to that obtained in similar conditions in mammals (Witt et al., 2000; Drenth et al., 2002; Ohmuraya et al., 2005), whereby gland cells and endodermal epithelial cells die through autodigestion, probably as a result from the lack of protection against digestive enzymes.

**Results**

The hydra Kazal1 gene belongs to the serine protease inhibitor Kazal family and is expressed in endodermal gland cells

Initially, a 486-bp-long Hv cDNA (Kazal1-486) was isolated, which encodes three KDs but lacks the 5’ end. Subsequently, two sequence tags (ESTs; ace_0078.y, ace_0064.y) expressed in H. magnipapillata and showing a perfect match with Kazal1-486 were obtained, thus allowed us to extend the Hv cDNA up to 569 bp (Fig. 1A, supplementary material Fig. S1). These cDNAs from two distinct hydra species comprise a single identical open reading frame, 504 bp long, leading to a 168 aa full-length putative translation product. The KD motifs are highly related to those found either in bilaterian serine protease inhibitors or in proteins like follistatin and agrin (Fig. 1B, supplementary material Fig. S2 and Table S1). In addition, we identified a signal peptide (probability, 0.998) with a cleavage site located between residues 16 and 17 (probability, 0.983), indicating that the Kazal1 protein is probably secreted.

In adult hydra, Kazal1 expression was restricted to the endodermal cell layer of the body column (Fig. 2A-C). In addition, the Kazal1-expressing cells exhibited the typical morphology of gland cells, with a multi-vacuolated cytoplasm corresponding to large secretory vacuoles (Fig. 2D-G, arrows). Those cells that are in direct contact with nutrients provide the proteolytic enzymes required for their digestion (Lentz, 1966). Similarly the hydra antistatin protease-inhibitor is expressed in gland cells (Holstein et al., 1992). Owing to its highly similar structure to protein inhibitors and its cellular localization, we speculated that Kazal1 might exhibit some protease inhibitory function such as to prevent the premature activation of digestive enzymes. Since Kazal1-expressing cells are immediately accessible to dsRNA when delivered by feeding, we established a feeding strategy to silence this gene through dsRNAi.

Ingestion of bacterially expressed dsRNAs is a harmless procedure in hydra

Hydra is a predator, feeding only on live, mobile animal preys and not on bacteria, we therefore adapted the solution used in planarians (Newmark et al., 2003). We converted bacteria to a sizeable food chunk by embedding them in agarose as part of an artificial food mix but, in addition, we added glutathione to the medium to trigger the hydra feeding-response, i.e. the mouth opening and the synchronized movements of the tentacles towards the mouth (Loomis, 1955). Finally, because hydra medium has extremely weak buffering capacities, we increased the buffer concentration to compensate, at least partially, the bacteria-induced acidity that prevented hydra from eating (Fig. 3A). This artificial food mix could actually be offered to hydra repeatedly over long periods without the appearance of unwanted toxic effects; populations treated over a month remained stable (Fig. 3B). Unc-22 dsRNA-treated control animals displayed a significant reduction of their size within the first two weeks of treatment, probably due to the change in diet. In fact, control hydra that were kept in the same conditions but were not exposed to agarose, showed a similar decrease in size (Fig. 3C).

We noticed rare cases (<1%) of heteromorphism as double-headed or double-footed hydra (not shown), which were similar whatever the type of dsRNA treatment, thus corresponding to non-specific effects linked to the feeding strategy. Finally, no significant variation in the head-regenerative abilities of hydra exposed once, three times, eight
times or 19 times to the *unc-22* dsRNA-bacteria-agarose mixture were noticed (Fig. 3D). Therefore, this treatment per se does not seem to affect the regeneration process.

The feeding strategy leads to a progressive and complete Kazal1 silencing

Few feedings were required to obtain an efficient silencing of the *Kazal1* gene in *H. vulgaris* because after three feedings, a noticeable difference was already seen between the hydra receiving *Kazal1* dsRNAs and those exposed to the control *unc-22* dsRNAs (Fig. 4A,B). After five feedings, *Kazal1*-expressing cells were no longer detected in the former, whereas *Kazal1* expression was not affected in the latter. When we examined the *Kazal1*-expressing cells under fluorescence microscope, few residual *Kazal1*-positive cells were nevertheless detected in the peduncle region, i.e. the lower part of the body column (Fig. 4C-E). Confocal imaging confirmed that the level of *Kazal1* expression in gland cells of *Kazal1*(-) hydra was either slightly residual or not detectable (Fig. 4H,I).

This silencing was specific as evidenced in *Kazal1*(-) hydra by the unaltered expression of *thrombospondin 1* (*Tsp1*, Fig. 4F,G), a gene normally expressed in the hypostome region (Miljkovic-Licina et al., 2004), or *cnox2*, a gene expressed in the neuronal cell lineages (M.M.-L., unpublished data). To test the duration of the feeding-induced RNAi, we treated hydra six times with *Kazal1* dsRNAs, and then left them to recover with normal feeding. Hydra remained completely devoid of *Kazal1* transcripts for at least nine days after the last RNAi feeding.
Autophagy in Kazal1-knock-down hydra (not shown). This value might, nevertheless, differ from one gene to another.

Kazal1 silencing affects the budding rate and is lethal

To analyze the fitness of Kazal1(–) hydra, we performed three distinct silencing experiments (RNAi-a, RNAi-b and RNAi-c) where 100, 60 and 65 H. magnipapillata respectively, were fed with bacteria producing either Kazal1 or empty vector dsRNA. When we compared the size of the Kazal1(–) hydra population to that of the control, we noticed a progressive but drastic decrease in the number of the Kazal1-treated hydra following the second dsRNAs exposure (Fig. 5A). After the fifth (RNAi-b and RNAi-c) and seventh time (RNAi-a) of exposure, the Kazal1(–) population size was approximately 60% that of the control. This evolution was affected by two distinct processes, first the budding process that produced new hydra during the first week and, second, the death rate of the animals noticed from the second week onwards. When we compared the different budding rates, i.e. the percentages of new animals produced upon budding in each experiment (Fig. 5B), we observed that the budding rate was significantly lower in Kazal1(–) hydra, i.e. 2.1, 3.6 and 3.3 times lower than in control hydra in RNAi-a, RNAi-b and RNAi-c experiments respectively. Although the budding rates were initially quite

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**Fig. 4.** Silencing of the Kazal1 gene in intact adult H. vulgaris.

(A-E,H,I) Kazal1 expression was detected after whole-mount in situ hybridization with (A,B) NBT-BCIP and (C-E,H,I) Fast-Red. A decrease in the level of Kazal1 expression was observed after 2 to 5 exposures to Kazal1 dsRNA (A,C,D), but not in hydra treated with (B) unc22 dsRNA or (E) agarose. (F,G) Tsp1 expression was not altered in hydra exposed 5x to Kazal1 dsRNA (upper) when compared to untreated animals (lower). Confocal views showing the residual or undetectable level of Kazal1 expression in gland cells of hydra exposed 5x to Kazal1 dsRNA (I) when compared to control hydra treated with agarose (H). DAPI staining (blue), α-tubulin staining (green). Bars: 150 µm (C-E,F), 4 µm (H,I).

**Fig. 5.** Survival and budding rates of Kazal1(–) H. magnipapillata.

(A) Sizes of the hydra populations exposed to Kazal1 dsRNAi. After each feeding hydra were counted and the percentage of Kazal1-silenced hydra over the control population exposed to the empty vector dsRNA was calculated. Each curve corresponds to a different experiment; 100, 60 and 60 hydra were initially taken for RNAi-a, RNAi-b and RNAi-c, respectively. (B) Budding rates observed in Kazal1(–) and control hydra populations in (a) RNAi-a, (b) RNAi-b and (c) RNAi-c experiments. The budding rate corresponds to the ratio between the number of new hydra produced by budding over the total number of hydra. Dark bars, control hydra; light bars, Kazal1(–) hydra. (C) Duplication of the peduncle and basal region observed in two different Kazal1(–) hydra after three feedings (RNAi-b).
similar in Kazal1-treated and control populations, budding rapidly disappeared after two days in Kazal1-treated populations, disappearing completely after either three (RNAi-b, -c) or four (RNAi-a) exposures. In control populations, however, budding was still going on after the fourth (RNAi-b, RNAi-c) and fifth (RNAi-a) feeding. Hence, the budding process was more intense and more prolonged in control hydra. In addition, the total number of Kazalli(−) hydra progressively decreased indicating that animals were dying, a process not observed in control populations. Therefore, Kazal1-silencing affects both, budding rate and animal survival. Since RNAi-a was prolonged to up to nine exposures with dsRNAs (20 days) and RNAi-b,c to up to seven exposures (15 days), we searched for possible morphological anomalies in Kazal1(−) hydra. Over this period (15-20 days), 11% Kazal1(−) hydra exhibited anomalies restricted to the peduncle and foot regions before dying during the following days (Fig. 5C). Those mutant animals displayed a very similar ‘clamp’-shape of their basal region, as if this region was undergoing duplication. This phenomenon started to appear after the third exposure in RNAI-b and RNAI-c but was not observed in the control populations.

Cellular disorganization of the gland cells in intact Kazal1(−) hydra

To analyze the cellular phenotype linked to Kazal1(−) silencing, we macerated some animals the day after each dsRNA exposure and performed immunocytochemistry with antibodies against ribosomal S6 kinase (RSK) and α–tubulin. In all cell types, staining for RSK was strong in the cytoplasm and uncovered the organization of the organelles (Chera et al., 2006). Among the various cell types, we did not detect any morphological modifications of the interstitial cells and their neuronal and nematocyte derivatives in Kazal1(−) hydra (data not shown). By contrast, when we analyzed the gland cells, we noticed a clear decrease in size until they eventually disappeared. When, very rarely, cells did survive a total of seven Kazal1(−) dsRNAs exposures, they exhibited fused vacuoles (Fig. 6A,B). In fact, we noted a progressively altered morphology of those Kazal1(−) gland cells: the cell outlines were often irregular showing notches, the nucleus was frequently no longer in its basal position, the graded distribution of the vacuoles along the cellular apico-basal axis (Lentz, 1966) was lost, while the vacuoles got progressively enlarged until they fused, forming huge vacuoles (Fig. 6A,D). The gland cells from control animals did not show any of those changes (Fig. 6B,C). To track cytoplasmic organelles in Kazal1(−) cells, we labeled those gland cells with anti-LBPA antibody, a specific marker of late endosomes (Kobayashi et al., 1998), and the mitotracker dye MitoFluor Red 589, which accumulates in mitochondria of live and fixed cells (Molecular Probes). We observed numerous cellular defects, like fusion of secretory vacuoles (Fig. 6Fb,c, arrowheads) and detachment of cytoplasmic parts from the apical pole as well as from the basal pole (Fig. 6Fb,c, arrowheads). In the latter case, those cytoplasmic parts occasionally contained mitochondria and could be as large as the apical moiety of the cell (Fig. 6Fd). However, nuclear fragmentation, which is a typical feature of apoptosis, was never detected. In hydra, gland cells differentiate from large dividing interstitial cells (Schmidt and David, 1986; Bode et al., 1987) and those cells were not affected when Kazal1(−) was silenced. Therefore, to quantify cell death of the gland cell population in Kazal1(−) hydra, we measured the percentage of gland cells over dividing interstitial cells in control and Kazal1(−) hydra at various time points in RNAI-a, RNAI-b and RNAI-c experiments (Fig. 6G). This ratio was stable in control hydra, varying between 90% to 100%. By contrast, it dropped drastically in Kazal1(−) hydra, down to 17% after the seventh exposure in RNAI-a, to 9% and 0% after the fifth exposure in RNAI-b and RNAI-c. Thus, we concluded that in the absence of Kazal1 expression, gland cells can neither differentiate nor survive. As previously noticed in the analysis of the population sizes and budding rates of Kazal1(−) hydra, the cellular phenotype was stronger in experiments RNAI-b and RNAI-c than in RNAI-a. In RNAI-a, the number of treated animals was larger suggesting that, animals were less homogenously exposed to the agarose-bacteria mixture and,

![Fig. 6. Cellular disorganization of gland cells in Kazal1(−) intact hydra.](image)

(A-F) Confocal images of H. magnipapilata gland cells exposed up to 7× to (A,D,F) Kazal1 or to (B,C,E) empty vector dsRNAs, subsequently immunodetected with (A,B) anti-RSK, (C,D) anti-α–tubulin, (E,F) 6C4 anti-LBPA antibodies (green) and stained with (E,F) Hoechst 33342 dye (blue) and the mitotracker dye MitoFluor Red 589. (F) Anomalies from four distinct gland cells; arrows indicate fused vacuoles, arrowheads cytoplasmic portions that are expelled from the cell. Bars, 10 μm (A-D), 5 μm (E,F).

(G) Evolution of the gland-cell index over the course of three independent RNAi experiments (RNAI-a, -b, -c). For each condition, the percentage of gland cells over doublets of dividing interstitial cells was measured in control and Kazal1(−) hydra. This percentage was stable in control hydra with a 90% to 100% value.
consequently, the Kazal1 gene was less efficiently silenced in these conditions.

**Cellular disorganization of the digestive cells in intact Kazal1(−) hydra**

Interestingly, we also noticed in Kazal1(−) hydra morphological alterations of the endodermal myoepithelial cells (also named digestive cells) (Fig. 7). Hydra digestive cells normally contain numerous digestive vacuoles that are predominantly located at the apical pole (Lentz, 1966). In both, Kazal1(−) and control hydra, digestive vacuoles detected with anti-RSK antibody were present until the fourth feeding and vanished at the subsequent stages (Fig. 7A,B, arrowheads).

However, from the third feeding onwards, we recorded in Kazal1-treated hydra, but not in control hydra, an increasing number of digestive cells that displayed large vacuoles (Fig. 7A,C,D,G,J arrows). In addition, from the fourth feeding, the basal pole of the Kazal1(−) digestive cells often detached, whereas the cell size drastically decreased (Fig. 7A,C). After seven Kazal1 dsRNA exposures, very few of those cells were actually able to support the maceration procedure and we observed mostly debris. Except gland cells, these alterations were not detected in any other cell types, including ectodermal epithelial cells (Fig. 7H). Concomitantly, we observed a progressive loss of the cytoplasmic organization, the tubulin network appearing more and more disorganized in cells where

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**Fig. 7.** Cellular disorganization of digestive cells in Kazal1(−) intact hydra. (A-G) Endodermal epithelial cells from *H. magnipapillata* (A,B,G) and *H. vulgaris* (C-F) exposed up to 7× either to (A,C,D,G) Kazal1 or to (B,E,F) empty vector dsRNAs, subsequently immunodetected with either the (A,B) anti-RSK, or the anti-α-tubulin (C-G) antibodies (green). Stars indicate phagosomes, arrowheads digestive vacuoles mostly abundant at the apical pole, arrows point to the unusual vacuoles that appear after 4× exposures to Kazal1 dsRNAs. (G) Two different confocal sections of a highly disorganized digestive cell that exhibits a giant vacuole (arrow), nu: nucleus. (H) Ectodermal epithelial cells of *H. vulgaris* exposed up to 5× either to Kazal1 dsRNAs immunodetected with the anti-α-tubulin antibody (green). Bars, 8 μm. (I,J) Endodermal epithelial cells from *H. magnipapillata* exposed 4× either to Kazal1 (J) or to the empty vector (I) dsRNAs, labeled with the late-endosome marker anti-LBPA antibody (green) and a mitotracker MitoFluor Red 589 (red). Arrows indicate mitochondria that are either (IJa) cytoplasmic or (Jb-j) intra-vacuolic; arrowheads show the disruption of the cellular basal pole in c, two vacuoles that undergo fusion in h. Bars, 10 μm (I, J,a-c,e,h,j); 2 μm in (Jd,f,g,i). In every panel, the cellular basal pole is at the top and nuclei were counterstained with Hoechst 33342 dye (blue).
either numerous or giant vacuoles were progressively occupying the cytoplasmic space (Fig. 7C,D,G).

However, like in gland cells, these vacuoles started to form without any sign of apoptosis or necrosis. Therefore, we suspected some autophagy process, whereby cytoplasmic organelles are sequestered in double-membrane vacuoles, the autophagosomes, which upon fusion with a late endosome will form an autolysosome, where degradation of the sequestered material will take place (Klionsky, 2005). To test whether those vacuoles were indeed containing cytoplasmic organelles, we performed a double labeling with the late endosome membrane marker 6C4 (Kobayashi et al., 1998) and a mitotracker dye MitoFluor Red 589 on cells isolated after four dsRNAs exposures. We noticed that most digestive cells from control hydra displayed a regular cytoplasmic distribution of mitochondria (Fig. 7I), some of them showing also few vacuoles that contained mitochondria (not shown). In Kazal1(−) hydra, by contrast, we detected very few digestive cells with clearly distinct cytoplasmic mitochondria (Fig. 7Jab), whereas in most cells the numerous vacuoles contained mitochondria that co-localized with the membrane marker of late endosomes (Fig. 7Jc-j, arrows). As previously noticed, many cells were loosing their basal moiety (Fig. 7Jc), whereas fusion of vacuoles was seen in others (Fig. 7Jh). The sum of these defects produced highly abnormal Kazal1(−) digestive cells that were very small in size and had no distinct cytoplasmic mitochondria but giant vacuoles (Fig. 7Jj). The presence of mitochondria, together with late endosomes in those numerous vacuoles indicated that they indeed contained cytoplasmic organelles and probably fused with lysosomes. Thus, these vacuoles, which we identified as autolysosomes, are the hallmark of a massive autophagy process. Hence, the depletion in the Kazal1 protease inhibitor, which is produced and secreted by the gland cells, also led to deleterious effects within the immediately surrounding cells, i.e. the digestive cells.

Kazal1 silencing does not affect kinetics of head regeneration
We then investigated, whether similar cellular modifications were also taking place in regenerating Kazal1(−) hydra. We first observed the modulations of Kazal1 expression after mid-gastric section: in wild-type hydra, we noticed an early decrease in the level of Kazal1 expression along the body column, but not in the regenerating stumps where the density of Kazal1-expressing cells remained high at the early and early-late stages of regeneration (Fig. 8A-C). A similar regulation was observed in control RNAi hydra (Fig. 8D and data not shown). By contrast, those animals exposed several times to Kazal1 dsRNAs exhibited complete silencing in the body column and a residual expression in the regenerating stumps as noticed after six feedings (Fig. 8E). Interestingly, intact hydra exposed to Kazal1 dsRNAs in the same experiment showed a complete silencing already after four feedings (Fig. 8F) suggesting that, silencing through RNA interference is indeed more efficient in the body column than in the regenerating tips. When head regeneration was tested in H. magnipapillata exposed up to five times to Kazal1 dsRNAs, tentacle rudiments started to appear without any delay (Fig. 8G and data not shown). However, a larger number of exposures to Kazal1 dsRNAs led to significant differences between the silenced and the control populations (Fig. 8H). After the seventh exposure, the appearance of tentacle rudiments was slightly delayed although the head regenerated normally. After the ninth exposure none of the silenced hydra regenerated because the bisection actually led to their death within the next 20 hours. These results indicate that, silencing Kazal1 expression in the body column and the regenerating stumps does not affect the head-formation process per se but rather the conditions that are necessary to survive the amputation stress. In fact, when silencing was maintained over a long period, the stress of the amputation became lethal.

**Fig. 8. Kazal1 silencing in regenerating hydra. (A-C) Kazal1 expression in wild-type regenerating hydra fixed 4, 6 and 30 hours after mid-gastric section. Notice the strong level of expression in the endodermal cells of the (A,B, arrowheads) foot-regenerating tips and (A,B,C, arrows) head-regenerating tips. (D,E) Kazal1 expression in H. magnipapillata having regenerated 4 hours is similar to the wild-type pattern in control hydra exposed 6× to (D) empty vector but strongly silenced after 6× exposures to (E) Kazal1 dsRNAs. The arrow and arrowhead indicate the residual expression in head- and foot-regenerating tips, respectively. (F) In intact hydra 4× exposures to Kazal1 dsRNAs (right) suffice to completely silence Kazal1 expression when compared with the control hydra exposed to empty vector (left). Bars, 150 μm. (G) Kinetics of tentacle rudiments appearance were similar in regenerating H. magnipapillata exposed 3× to unc-22 (□) or to Kazal1 (●) dsRNAs. (H) Appearance of tentacle rudiments recorded 42 hours (left) and 44 hours (right) after mid-gastric section of hydra exposed up to 9× either to empty vector (□) or to the Kazal1 (●) dsRNAs.
Enhancement of the vacuolization phenotype in Kazal1(–) head-regenerating tips

To analyze the morphology of the Kazal1(–) cells located in the regenerating tips, we bisected hydra after three exposures to Kazal1 dsRNAs, and sliced-out the regenerating tips one or four hours later to provide single cell preparations subsequently submitted to anti-hyCREB and anti-RSK immunocytochemistry (Fig. 9A,B). The anti-hyCREB antibody provides a strong and specific nuclear signal in most hydra cell types, i.e., epithelial cells, neurons, interstitial cells and a cytoplasmic signal in gland cells and dividing nematoblasts (Chera et al., 2006). Moreover, the CREB protein becomes transiently cytoplasmic and its level is dramatically enhanced in stress conditions, such as immediately after amputation (S.C., unpublished observation). In Kazal1(–) hydra, the morphology of the ectodermal epithelial cells, interstitial cells, neurons and nematoblasts, prepared from either head- or foot-regenerating tips, was not altered when compared with control hydra (Fig. 9A and data not shown). By contrast, only a very small number of gland cells was found in macerations of regenerating Kazal1(–) hydra. They all exhibited the usual enhanced cytoplasmic CREB staining at that stage of regeneration, large vacuoles and also a cellular disorganization similar to that observed in intact Kazal1(–) hydra after five exposures (compare Fig. 9Ad,i,n with Fig. 6D).

In addition, we noticed that a large number of Kazal1(–) digestive cells prepared from both head- and foot-regenerating tips contained large cytoplasmic vacuoles and showed an enhanced CREB cytoplasmic staining (Fig. 9Aa,f,k, arrows). We identified three progressive stages in this Kazal1(–) phenotype of digestive cells (Fig. 9B), starting from stage 0, which corresponds to normal endodermal epithelial cells. At stage 1, one or several large vacuoles were present in the vicinity of the nucleus. At stage 2, additional vacuoles were dispersed in the cytoplasm, whereas at stage 3 those vacuoles were fused, occupying most of the cytoplasmic space. Over 98% of the digestive cells isolated from control hydra looked normal (like in stage 0) at both times of regeneration (Fig. 9C and supplementary material Table S2). However, as anticipated, Kazal1(–) cells isolated from head-regenerating tips were strongly affected, to a much larger extent than those prepared from foot-regenerating tips. One hour after bisection, stages 2 plus 3 were the most frequent with over 60% and 11%, respectively. In both contexts, these alterations were transiently observed, as 4 hours after bisection, only 18% and 3% of the cells from head- and foot-regenerating tips, respectively, were still strongly affected (stages 2 plus 3). Therefore, silencing of the Kazal1 gene induced drastic cellular alterations in regenerating tips, a phenotype that is dramatically enhanced upon amputation stress when compared with intact Kazal1(–) hydra, and far more severe in head- than in foot-regenerating tips.

Discussion

Ingestion of bacterially-expressed dsRNAs leads to a progressive, efficient and reversible gene silencing in hydra.

We show here, that repeated feedings with dsRNA-producing bacteria specifically and efficiently suppresses gene expression
in hydra below the in situ hybridization detection limit. In case of the Kazall gene, five feedings seemed to be necessary in *H. magnipapillata*, although three were sufficient in *H. vulgaris* to detect the cellular phenotype. In addition to species sensitivity, efficiency of the RNAi procedure is probably linked to the size of the initial animal population, as evidenced by the difference in the respective intensities of the phenotypes observed in RNAI-a versus RNAI-b-c experiments. In fact, repeated feedings were required to obtain the phenotype described here, but single or only few feedings can suffice to generate phenotypes with more critical genes (S.C., unpublished data) and, indeed, incremental numbers of feedings can be used to generate pseudo-‘allelic series’. We also noticed that Kazall expression was more robust in regenerating tips than in the body column, implying that specific spatio-temporal regulations probably modulate the sensitivity to silencing through RNAI.

Although this novel method was designed to damage hydra as little as possible, it is not completely harmless. For each RNAi treatment hydra have to stay for 1 hour in an hyperosmotic environment and are submitted to additional washes over the normal culture procedure, which can lead to the loss of the weakest hydra. We, nonetheless, did not notice any dissociation event or bacterial infection. Agarose sieving should be fine because hydra that ingest a too large piece of agarose cannot spit it out the following days, become unable to eat artemia and eventually die. These adverse effects could account for the fact that the treated populations exhibited some rare nonspecific cases of heteromorphosis. But stepwise, specific and reproducible phenotypes were generated with several different genes at the cellular and also the developmental level (this work and our unpublished data), indicating that the nonspecific effects did not mask their appearance.

Finally, with this method we were able to silence genes expressed in the ectoderm, thereby demonstrating that the silencing is systemic and not restricted to the place of delivery (R.d.R., S.C., M.M.L., unpublished observations). Hence, this procedure appears to be nontoxic with only moderate side effects and offers a good starting point to set up large-scale functional screens in hydra, using for example the growing EST complement available. RNAI by bacterial feeding is an extremely cheap and simple method, requiring no additional specialised material besides the classical hydra culture and basic molecular biology laboratory equipment. Therefore, hydra could be subjected to genome-wide functional screens that would identify the hydra genes required for their amazing cell plasticity and regenerative potential, as it has recently been done in planarians (Reddien et al., 2005).

**Kazall silencing leads to autophagy in gland cells and epithelial digestive cells**

Kazall(–) silencing had dramatic effects on hydra at the homeostatic, morphological and cellular level. Electronic microscopy studies allowed a precise description of the cellular features of the hydra gastroepidermis. It is made up of two endodermal cell types, the gland cells, which produce and release zymogens in the gastric cavity, and the myoepithelial endodermal cells, which carry out the digestive function (Lentz, 1966). Mature gland cells, which are abundant along the body column, display numerous similarities with pancreatic acinar exocrine cells. In both cases, cells are packed with secretory vacuoles that become larger at the apical pole. Secretion is of the apocrine type, the content of these vacuoles being pinched off from the apical pole of the cell into the digestive cavity after feeding. In hydra, the digestive cells are large columnar myoepithelial cells that connect to the mesoglea through their basal pole and extend microvilli and flagella into the gastric cavity. Those cells display a very heterogeneous content of membrane-bound structures, including digestive vacuoles mostly seen in the apical region, residual bodies that contain irregular structures and are mostly found at the basal pole of starved hydra, and an intracellular space that is frequently observed but rather small in the digestive cells located along the body column (Lentz, 1966). Moreover, DNA-containing phagocytic vacuoles or phagosomes, were described when the cells carry out macrophagic functions. These vacuoles or phagosomes are more abundant in starved than in well-fed animals suggesting that, phagocytosis of neighboring cells is a survival mechanism in hydra (Bosch and David, 1984). In intact Kazall(–) hydra, both cell types involved in the digestive process showed progressive dramatic changes.

The gland cells first exhibited an altered apico-basal organization together with an enlargement of their vacuoles, which subsequently fused. Thereby, after four or five dsRNAi exposures, they formed huge vacuoles and expelled part of their cytoplasmic content at a time when the gland cells underwent a phase of massive cell death. Concerning the epithelial digestive cells, we did not notice any modification of their apical digestive vacuoles after several exposures to dsRNAs. Subsequently, the digestive process was seemingly less active in control and also in Kazall(–) hydra, as evidenced by the limited number of digestive vacuoles after five feedings. However, at the same time, we observed in Kazall(–) hydra large membrane-bound vacuoles that appeared before the digestive cells started to lose their basal part, shrink and eventually die. These unusual vacuoles that are located at variable positions along the cellular apico-basal axis, contained in most cases mitochondria associated with late-endosome membranes and, more rarely, non-polymerized tubulin associated with DNA.

To carry out their digestive function, digestive cells are permanently producing membrane-bound vacuoles through pinocytosis and phagocytosis. Nevertheless, in Kazall(–) hydra, the presence of mitochondria and late endosomes within most of these vacuoles indicate that the membrane-bound vacuoles presumably correspond to autophagosomes and autolysosomes, which subsequently fuse and ultimately disrupt their membranes to release their content into the intracytoplasmic space. Formation of autophagosomes, which correspond to large autophagic vacuoles fused to lysosomes, is the dynamic process that characterizes autophagy, a cell-death mechanism distinct from necrosis and apoptosis (Lockshin and Zakeri, 2004). In hydra, in conditions where we noticed this vacuolization phenotype, we did not record any swelling of cell membranes and organelles (a hallmark of necrosis); neither did we observe any specific signs of apoptosis such as nuclear fragmentation or cell shrinkage. Hence, the cellular anomalies induced upon Kazall silencing display the characteristic features of autophagy, a process that probably accounts for the massive cell death of gland and
digestive cells as well as the high rate of mortality observed in Kazal1(−) hydra.

Amputation stress enhances the Kazal1(−) autophagy phenotype
In regenerating Kazal1(−) hydra, very few gland cells survived the amputation stress. The very small number of gland cells that did survive were highly disorganized, with enlarged vacuoles and a phenotype more pronounced in cells from head-regenerating tips. Hence, compared with intact Kazal1(−) hydra, the vacuolated lethal phenotype of gland cells was dramatically increased upon amputation. Similarly, immediately after bisection, over 30% of the digestive cells prepared from head-regenerating halves formed large membrane-bound intracellular spaces, which are usually only observed in endodermal epithelial cells of the tentacles. Since head-regenerating halves do not contain any tentacles, we conclude that this cellular phenotype is linked to Kazal silencing.

This enhanced cellular digestive phenotype, observed within the first hours after amputation, was transient when only three Kazal dsRNA exposures had taken place. This transient feature can be explained in two ways. Either the observed cellular are reversible and quickly fixed by the affected digestive cells, or the damaged cells are replaced by healthy ones. We favor the second scenario because on one hand, we expected the amputation stress to be rather limited (affecting only the endodermal cells located in the regenerating tip where the cellular consequences of amputation are major; S.C., unpublished data), on the other hand, we showed that silencing was incomplete in the regenerating tips after three dsRNA exposures. However, after a higher number of dsRNA exposures inducing a prolonged Kazal silencing, the cellular modifications were probably no longer reversible, as evidenced by the fact that the amputation stress became lethal in Kazal1(−) hydra after seven exposures to dsRNAs.

Interestingly, the immediate enhancement of the cellular phenotype after bisection was strongly asymmetric. We observed major modifications of gland cells and endodermal epithelial cells in the head-regenerating stumps but not in foot-regenerating ones. An asymmetric response during early regeneration has already been reported at the gene expression level (Gauchat et al., 1998) and also at the signal transduction level (Cardenas and Salgado, 2003; Kaloulis et al., 2004), but this is the first report of an immediate asymmetry in the cellular response to the amputation stress.

The hydra Kazal1(−) phenotype mimics the Spink1/Spink3 autophagy pancreatic phenotype
Given the structure of the Kazal1 protein and its specific production by the gland cells, we conclude that Kazal1 functions as a protease inhibitor, and thus propose that the Kazal1(−) phenotype is caused by the premature activation of secreted digestive enzymes, a process normally prevented by Kazal1. This situation is somewhat analogous to the effect of mutations in the human pancreatic trypsin inhibitor SPINK1 (Witt et al., 2000, Drenth et al., 2000) or the mouse cognate gene Spink3 (Ohmuraya et al., 2005). It has been proposed that Kazal-type serine protease inhibitors like SPINK1 and SPINK3 prevent the intra-pancreatic activation of trypsinogen (Witt et al., 2000); when deficient, the imbalance between trypsinogen activation and protease inhibitors leads to enzymatic activation, autodigestion of the exocrine pancreas by activated proteases and progressively chronic pancreatitis characterized by the loss of acinar cell mass. Pancreatic acinar cells of Spink3 mutant mice start forming vacuoles immediately after birth, which have been identified as autophagosomes (Ohmuraya et al., 2005). Similarly to the hydra, where gland cells die in few days, the massive cell death observed in pancreatic acini was associated neither with necrosis nor with apoptosis, but rather with autophagy as confirmed by histological and biochemical evidences (Ohmuraya et al., 2005). Just before cell death, disruption of the membranes of autophagosomes and the subsequent release of their enzymatic content within acinar cells were observed. Interestingly, Spink3−/− mice not only exhibit an autophagic degeneration of the exocrine pancreas but a concomitant degeneration of the thin smooth-muscle layer, and the villi of the duodenum and the small intestine, and altogether, those defects lead to severe growth retardation and animal death.

Furthermore, beside the essential roles of SPINK3 in the maintenance and regeneration of acinar cells in the perinatal period, a cytoprotective function was also identified in the adult mouse pancreas (Neuschwander-Tetri et al., 2004). Pancreatic injury in mice induces specific overexpression of Spink3, an upregulation that might reflect an endogenous cytoprotective mechanism to prevent further injury. In hydra, the specific maintenance of high levels of Kazal1 expression in regenerating stumps, and the dramatic cellular effects observed when Kazal1 was silenced, support a similar cytoprotective role for Kazal1 in regenerating hydra. Hence, this functional study indicates that Kazal1 has indeed a key role in the digestive physiology of hydra, the maintenance of homeostatic conditions that allow growth and survival of hydra, but also the protection of those cells exposed to the amputation stress. In fact, such cytoprotective function that we have highlighted during regeneration has never been reported in any previous studies concerning hydra regeneration but appears as a specific requirement of the regeneration process per se.

Regulation of autophagy from hydra to mammals
Autophagy is a well-conserved process, initially characterized as a response to starvation and growth factor deprivation to promote cell survival. The genetic machinery at work during this process has been genetically dissected in yeast, Dictyostelium discoideum, nematode and Drosophila melanogaster (Levine and Klionsky, 2004). However, the regulation of this process in pathological conditions is still poorly understood. The protective function of Kazal-type serine protease inhibitors against excessive autophagy was previously suspected from the physiopathological alterations of the exocrine pancreas linked to human mutations of the SPINK1 gene. It recently received strong support from detailed cellular and molecular analyses of mice with a mutated version of the homologous Spink3 gene. The results reported here provide a first example of a genetic defect affecting an evolutionarily-conserved gene, which leads to similar cellular alterations, i.e. excessive autophagy in hydra and mammals. This suggests that hydra provides a useful model system to highlight some evolutionarily-conserved aspects in the regulation of autophagy.
Materials and Methods

Hydra culture

H. vulgaris (Hv, Zürich strain) and H. magnipapillata (Hm, strain 105) were cultured in hydra medium (HM; 1 mM NaCl, 1 mM CaCl2, 0.1 mM KCl, 0.1 mM MgSO4, 1 mM Tris pH 7.6) as in Gauchat et al. (Gauchat et al., 2004). For regeneration experiments, hydra were bisected 24 hours after the last RNAi feeding and fixed at various time points of regeneration.

Cloning of the hydra Kazal1 gene

Primer GlI-f1ca (tgacaggtctgtgctggttgctg) and Gli-rev2c (caagadcarytwgthcayca) were used to amplify Kazal1 cDNA sequences from H. magnipapillata. The Kazal1-486 riboprobe was subsequently hybridized and quantified (supplementary material Fig. S2).

In situ hybridization coupled to immunohistochemistry

The Kazal-486 riboprobe was SP6 synthesized in and in situ hybridization was performed as in Gauchat et al. (Gauchat et al., 2004). Samples were stained with either NBT/BCIP or Fast Red (Daco Cytometry), then washed in PBST, blocked in 3.5% BSA, incubated overnight in anti-actin-antibody (1:500, Sigma), washed in PBST, incubated 3 hours at RT in Alexa Fluor 488 anti-mouse antibody (1:500, Molecular Probes), washed again in PBST, mounted in Mowiol and examined on an Axioskop (Zeiss) microscope equipped with a Plan-Apochromat 40x objective. Images were captured through an Axiozoom camera with the Openlab System Software (Improvision), processed with the FotoStation Pro4.5 software (FotoWare) and final artwork was done with Photoshop software (Adobe). Confocal imaging was performed on a TCS SP2 AOBIS Leica microscope.


