Molecular cloning and cellular distribution of two 14-3-3 isoforms from Hydra: 14-3-3 proteins respond to starvation and bind to phosphorylated targets

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Abstract

In the simple metazoan Hydra a clear link between food supply and cell survival has been established. Whilst in plants 14-3-3 proteins are found to be involved in signalling cascades that regulate metabolism, in animals they have been shown to participate in cell survival pathways. In order to explore the possibility that 14-3-3 proteins in Hydra could be involved in regulating metabolism under different conditions of food supply, we have cloned two isoforms of 14-3-3 proteins. We show here that 14-3-3 proteins bind to phosphorylated targets in Hydra and form homo- and heterodimers in vitro. 14-3-3 proteins are localised in the cytoplasm of all cells and also in the nuclei of some epithelial cells. This nuclear localisation becomes more prominent during starvation. Moreover, 14-3-3 protein is present in large amounts in food granules and from this we conclude that it performs functions which are associated with metabolism and food storage in Hydra.

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Introduction

14-3-3 proteins are a family of highly conserved small acidic proteins that bind to phosphoserine residues within an amino acid consensus site of their target proteins (reviewed in [1]). Binding to 14-3-3 after phosphorylation of specific serine residues can regulate the activity of 14-3-3 targets in three different ways. Enzymes that regulate plant metabolism in response to environmental stimuli, for instance, can be either inhibited or activated (e.g., nitratreductase, sucrose phosphate synthase; reviewed in [2]). A second possibility is that 14-3-3 binding changes the stability of its binding partner (e.g., Raf-1, [3]) or protects it from proteolysis [4]. The third mode of regulation exerted by 14-3-3 proteins is the alteration of the subcellular distribution of its targets. After DNA damage in yeast checkpoint kinase 1 phosphorylates Cdc25. A 14-3-3 homolog, rad 25, then associates with Cdc25 and prevents its translocation to the nucleus. As a result Cdc25 cannot dephosphorylate Cdc2 and promote G2/M entry. This leads to DNA damage-induced G2 arrest [5]. In mammalian cells in response to cell survival factors such as interleukin 3 (IL-3), the proapoptotic bcl-2 family member BAD is phosphorylated at Ser 136 by the IL-3-responsive kinase Akt. In this state it binds to 14-3-3, which prevents BAD from interacting with Bcl-XL in the mitochondrial membrane and thus triggering apoptosis [6].

Although it appears that 14-3-3 target proteins are quite diverse, they are generally proteins involved in cell cycle progression, survival pathways, and metabolic control (reviewed in [7]). In Hydra a clear link has been described between metabolic state (or food supply) and cell survival. In well-fed Hydra cell numbers double every 2 to 3 days. After the animals reach a certain size, excess cells are used for budding. Under conditions of starvation, on the other hand, no such increase in cell numbers occurs [8]. The rate of cell proliferation, however, indicates that cell divisions occur at roughly the same rate independent of the supply of nutrients, thus leading to production of “excess” cells. These then undergo apoptosis and are phagocytosed by their neighbours.
The factors that regulate cell survival under varying conditions of food supply in *Hydra* are currently not known. It seems likely, however, that cell–cell communication via secreted factors and signalling cascades are required. To explore the possibility that 14-3-3 proteins are involved in signalling cascades that regulate cell survival and metabolism under different conditions of food supply, we have cloned two isoforms of 14-3-3 proteins from *Hydra* and analysed their cellular distribution. We have also shown that the two isoforms can dimerise and that 14-3-3 binds to phosphorylated target proteins from *Hydra* extracts.

**Material and methods**

*Hydra culture*

*Hydra vulgaris* was cultured at a temperature of 18°C in medium containing 0.1 mM KCl, 1 mM NaCl, 0.1 mM MgSO₄, 1 mM Tris, and 1 mM CaCl₂. The animals were fed regularly with freshly hatched *Artemia nauplii*.

**RT-PCR and cDNA library screen**

For RT-PCR degenerated primers of the following sequence were used: 5’ Primer, 5’-AA(ACT) (CT)T(ACT) (AT) (GC)(ACT) (AG) (AT) (ACGT) AT(ACGT) A(AG) (ACGT) GT(ACGT) GC(ACGT) GT(ACGT) TA(CT) AA(AG) AA(CT)-3’ and 3’ Primer, 5’-TGC AT (AGT) AT(ACT) A(AG) (ACGT) (AG) (ACGT) GT(ACGT) (GC)(AT)(AG) TC(CT) TT(AG) TA -3’. The PCR products were cloned into pCR 2.1 TOPO-vector (Invitrogen) and sequenced. The inserts were used to produce DIG-labelled probes to screen the cDNA library as described previously [9].

**Alignments and phylogenetic tree construction**

14-3-3 sequences were aligned using Clustal W. The following 14-3-3 proteins were included (names with GenBank accession numbers): *Entamoeba* 1, P42649; *Entamoeba* 2 AAA80186; *Entamoeba* 3 P42648; *Dictyostelium* P 54632; *Chlamydomonas* P 52908; *Arabidopsis*: μ NP 565977, ε NM 102411.1, δ NP 564167.1, ω NM 103213.1, ϕ NM 106479.1, χ NM 116969.1, ν NM 121610.1, γ NM 111119.1, β NM 123029.1, k NM 25941.1; *Homo* ε NP 006752; *Xenopus* ε AAC 41251.1; *Drosophila* ε P 92177; BMH 1 NP 011104.1, BMH 2, NP 010384.1; rad 24 NP 594167; rad 25 NP 594247; *Homo* σ NP 006133.1, Mus σ NP 061224.1, *Homo* γ XP 050409, *Homo* η S 38532, Mus η BAA 13422, *Homo* τ NP 006817.1, Mus τ NM 011739.1, *Homo* β NP 003395.1, *Rattus* β NP 062250.1, *Homo* γ XP 088665, Mus γ JC 3584, *Xenopus* γ AAC 41252.1, *Drosophila* γ NP 476884.1, *Caenorhabditis* flt 1 NP 502235, flt 2 NP 509939, *Schistosoma* Q 26540, *Hydra* 14-3-3HyA AY162284. The tree was constructed using Treeview software.

**Whole-mount in situ hybridization**

Whole-mount in situ hybridization was carried out as described previously [10].

**Northern blot analysis**

*Hydra* mRNA was isolated using the mRNA purification kit (Amersham). The hybridization step with a DIG-labelled RNA probe was carried out at 60°C for 12 h.

**Elutriation**

A total of 2000 animals were agitated for 3 to 4 h in dissociation medium containing 8 mg/ml pronase E (FLUKA). They were then centrifuged at 800 rpm for 5 min. The supernatant was again centrifuged at 1300 rpm for 5 min. Both pellets were dissolved in dissociation medium and filtered. The cell suspension was then introduced into the elutriation chamber and centrifuged at velocities from 3800 rpm (F1) to 1100 rpm (F8). Fractions of 100 ml were collected at a flow rate of 15 ml/min for F1–F7 and F8 at 30 ml/min. These fractions were then centrifuged again: F1 and F2 at 1500 rpm for 10 min and F3–F8 at 800 rpm for 10 min, 4°C. Cell pellets were examined microscopically for their contents (see Fig. 3b) and then dissolved in SDS sample buffer, boiled for 5 min, and used in SDS–PAGE.

**Subcellular fractionation**

One thousand hydra were suspended in 2 ml buffer M (250 mM sucrose, 5 mM Tris/HCl, pH 7.5, 10 μg/ml antipain, protease-inhibitor cocktail complete (Roche), 1:100 diluted phosphatase-inhibitor I and II (Sigma), 4 mM pefabloc, 10 μg/ml pepstatin A, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM vanadate), and Dounce homogenised with 70 to 100 strokes. Cell breakage was checked microscopically. The homogenate was then centrifuged at 800g for 15 min. The pellet was washed in buffer M containing 0.7% NP-40. This was the nuclear fraction. The 800g supernatant was centrifuged again at 17,000g for 30 min to reveal the mitochondrial pellet. The supernatant was centrifuged at 100,000g for 60 min. This centrifugation resulted in the microsomal pellet and the cytoplasmic supernatant.

All fractions were equalised for protein content before SDS–PAGE.

**Western blot analysis**

Protein fractions were separated on a 12% SDS–PAGE. The separated proteins were transferred to immobilon P membrane (Millipore). The membrane was probed with the K19 antibody (Santa Cruz) as recommended by the manu-
Cloning of two 14-3-3 isoforms from Hydra

We designed degenerated primers to highly conserved regions of the 14-3-3 sequence. Touchdown PCR using these primers yielded a major product of the expected size (600 bp) as well as some less abundant smaller fragments. These were cloned into pCR 2.1 TOPO and sequenced. Two different sequences which both showed high homology to 14-3-3 from mammals were obtained. They were used to screen a Hydra cDNA library for full-length clones. Fig. 1 shows a sequence alignment of the two isoforms of Hydra 14-3-3, which we designated 14-3-3 Hy A and 14-3-3 Hy B, with 14-3-3 proteins from yeast (Saccharomyces BMH1 and BMH2), Schizosaccharomyces rad 24 and rad 25, plants (Arabidopsis), the protozoan Entamoeba, Caenorhabditis, Drosophila, and two vertebrates (Rattus 14-3-3 ε and Homo 14-3-3 e).

14-3-3 function depends on the formation of nine α-helices (Fig. 1, grey boxes). In 14-3-3 dimers these helices form a palisade around an amphipathic groove which constitutes the binding site for 14-3-3 target proteins. The residues lining the groove are highly conserved between species. These invariant residues are also conserved in both isoforms of Hydra 14-3-3. They include three leucines on the hydrophobic side: L 189, L 237, and L 244 (Fig. 1, boldface). On the basic side the invariant residues K 54, R 61, and R 65 are also present in the Hydra 14-3-3 clones (Fig. 1, boldface). The helix α 5 has polar groups belonging to the conserved amino acids K 137, D 141, R 144, and Y 145 (Fig. 1, boldface). 14-3-3 binding to phosphoserine is probably mediated by the cluster of basic amino acids R 61, R 65, K 137, and R 144 on the basic face of the groove (Fig. 1, boldface).

Most 14-3-3 proteins contain an annexin-like consensus sequence (18MKGDYYRYLAETGD154), which has been suggested to be involved in 14-3-3-stimulated exocytosis in bovine adrenal chromaffin cells [13]. Interestingly, the G152, printed in boldface, which is present in all 14-3-3 clones except for 14-3-3 from Entamoeba and one isoform from Caenorhabditis, is also lacking in both Hydra 14-3-3 isoforms (Fig. 1, arrow).

To compare the two new Hydra 14-3-3 proteins with different isoforms of 14-3-3 found in higher animals and in plants, we constructed the unrooted phylogenetic tree size ranging from 195 to 49 nm depending on the selected zoom factor. The axial distance between optical sections was 200 nm for zoom factor 4 and 1 μm for zoom factor 1. To obtain an improved signal-to-noise ratio each section image was averaged from four successive scans. The 8-bit greyscale single-channel images were overlaid to an RGB image assigning a false colour to each channel, and then assembled into tables using Adobe Photoshop 5.5.
shown in Fig. 2. We included ε, β, τ, η, γ, α, and ε isoforms from vertebrates, most of the designated isoforms from Arabidopsis that could be found in the database, and the unicellular algae Chlamydomonas, and also the known 14-3-3 genes from Dictyostelium, yeast, Entamoeba, Caenorhabditis, Schistosoma, and Drosophila.

The tree resembles results of previous phylogenetic analyses that included 125 14-3-3 sequences from 48 species [14,15]. The two new Hydra isoforms are found at the base of the main metazoan branch. They do not fall into any of the established isoform groups and are also very distinct from each other.

Northern blot analysis showed that both 14-3-3 genes are expressed in Hydra, 14-3-3 Hy A at a higher level than 14-3-3 Hy B. For 14-3-3 Hy B two transcripts were detected at sizes of 1.35 and 1.8 kb. For 14-3-3 Hy A three transcripts were observed at sizes of 1.2, 1.7, and ca. 2.0 kb (not shown). Multiple transcripts for 14-3-3 proteins have also been reported from Caenorhabditis and from Drosophila, where they were attributed to varying 3′ UTRs [16,17].
In situ hybridisation experiments showed transcripts for both 14-3-3 isotypes in all cell types and in every part of the animals. Whereas 14-3-3 Hy A was evenly distributed between the two epithelial layers, 14-3-3 Hy B expression appeared slightly stronger in the endoderm (not shown).

Hydra 14-3-3 protein is present in all cell types

The polyclonal rabbit antibody K19 (Santa Cruz), which reacts with 14-3-3 proteins from most species, was used to analyse hydra cell lysates by SDS–PAGE and Western blotting. Fig. 3a, lane 1(Hy), shows a Western blot of hydra tissue dissolved in SDS sample buffer and separated on SDS–PAGE. Two 14-3-3 bands at 32 and 33.5 kDa were clearly visible. These could reflect the cloned Hydra 14-3-3 isoforms. 14-3-3 Hy A has a theoretical molecular weight of 28.6 kDa, 14-3-3 Hy B of 28.1 kDa.

_Hydra_ consists of endodermal and ectodermal epithelial cells and of interstitial cells that are situated in interstitial spaces between the cells in the epithelial layer. To analyse 14-3-3 in different _Hydra_ cell types we separated dissociated hydra cells by elutriation. Elutriation separates cells on the basis of their size [18]. Separation of the different cell types is not complete, however. The composition of the seven cell fractions we analysed is shown in Fig. 3b. 14-3-3 protein was present in all fractions (F2–F8) (Fig. 3a). The two bands shown in lane Hy, however, were not uniformly present in fractions containing different cell types. The faster migrating band was absent from F4 and F5, but present in F2 and F3. An additional weak band at 35 kDa

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Fig. 3. (a) Western blot with fractions of whole hydra lysates (hy) and separated hydra cells. Cells were separated by elutriation. This revealed fractions F2–F8. The content of fractions F2 to F8 was determined microscopically and is shown in (b).
was observed in F2, F3, F7, and F8. The two fractions F4 and F5, which only have the 33.5-kDa band, contain ectodermal epithelial cells, gland cells, and nematocytes.

**Hydra 14-3-3 proteins are localised in the cytoplasm and in particle fractions**

We examined the subcellular distribution of 14-3-3 protein in cell lysates. Hydra were Dounce homogenised in isotonic buffer and subjected to differential centrifugation to yield fractions containing nuclei, mitochondria, microsomes, and cytoplasm. Fig. 4 shows that three bands with molecular weights of 32, 33.5, and 35 kDa, representing 14-3-3 proteins, were present. The 35-kDa band was lacking in the microsomes. The additional band in the mitochondrial fraction at 40 kDa is only observed in some preparations and seems to be nonspecific. The most prominent band was again the 33.5-kDa band. These results indicate that 14-3-3 proteins in *Hydra* are not only cytoplasmic, but are also present in particle fractions and especially in the nucleus. In order to define the exact subcellular localisation of 14-3-3 protein we resorted to immunocytochemistry (see below).

We speculated that the three 14-3-3 bands could reflect differential migration of 14-3-3 isoforms from *Hydra*. In order to investigate this question further, we expressed both isoforms as N-terminally His-tagged fusion proteins in bacteria.

Fig. 5 shows Coomassie staining of His-tagged 14-3-3 Hy A and Hy B expressed in *E. coli* after purification on a nickel column. Both isoforms were recognised by the K19 antibody and have approximately the same migration behaviour on PAGE. This indicates that the K19 antibody does not distinguish between the two 14-3-3 isoforms in *Hydra*. Hence, the 14-3-3 signals we obtained on Western blots described above and in immunocytochemistry (see later) include both 14-3-3 isoforms.

**Hydra 14-3-3 isoforms can form homo- and heterodimers in Far Western Overlay assays**

14-3-3 proteins are known to dimerise, and a dimer of two 14-3-3 molecules is required to bind phosphorylated target proteins. It has been shown that heterodimers between different isoforms can also form [19]. We asked whether this would occur between the two *Hydra* isoforms and if we could obtain means to distinguish the two isoforms from each other. Purified His-tagged 14-3-3 Hy A and 14-3-3 Hy B were labelled with digoxigenin and used to probe a
Western blot containing unlabelled recombinant His-tagged 14-3-3 (Far Western overlay assay, described in [11]). When DIG-labelled 14-3-3 Hy A was used to probe a Western blot with 14-3-3 proteins expressed from the plasmid pRSET in E. coli, 14-3-3 Hy A and 14-3-3 Hy B were both recognised. 14-3-3 Hy A bound more strongly to itself than to 14-3-3 Hy B (Fig. 6a). On the other hand, when 14-3-3 Hy B was used to probe the Western blot, it bound only to 14-3-3 Hy A, not to itself.

In order to find out whether DIG-labelled Hydra 14-3-3 proteins also interacted with endogenous proteins from Hydra, we probed lysates with DIG-labelled 14-3-3 Hy A and 14-3-3 Hy B. 14-3-3 Hy A revealed three very strong bands. The lower two bands correspond to the 32- and 33.5-kDa K19 signals shown in Fig. 3 and 4. DIG 14-3-3 Hy B, on the other hand, only recognised the 33.5-kDa band (Fig. 6b). As the DIG-labelled Hy B probe was not able to interact with recombinant Hy B in the overlay shown in Fig. 6a, but could interact with Hy A, it is tempting to speculate that the 33.5-kDa K19 band corresponds to 14-3-3 Hy A. The nature of the 32- and 35-kDa bands, however, cannot be deter-
mine.

When we loaded very large amounts of hydra lysate in overlay experiments a number of weaker bands in the molecular weight range between 50 and 100 kDa could also be observed (not shown). These appeared to be 14-3-3 target proteins. Since our DIG-labelled 14-3-3 Hy A and Hy B probes did not yield strong signals with such target proteins, we turned to using yeast 14-3-3 (BMH 1) as a probe.

**Phosphorylated targets for 14-3-3 binding are present in Hydra lysates**

When lysates from two different species of Hydra, Hydra vulgaris and Hydra magnipapillata, were separated on SDS–PAGE, Western blotted, and probed with the yeast 14-3-3 probe, six prominent bands could be observed. They correspond to molecular weights of approximately 118, 116 (114 in H. magnipapillata), 90, 75, 70, and 65 kDa (Fig. 7, left panel). A 14-3-3 blocking phosphopeptide of the sequence ARAAS(P)APA, corresponding to the consensus target sequence for 14-3-3, abolished all 14-3-3 binding, except for some residual binding to the 116-kDa protein (114 kDa in H. magnipapillata, respectively; Fig. 7, right panel).
To test if these interactions were phosphorylation-dependent we incubated the lysates for 1 h at room temperature in the presence or absence of phosphatase inhibitors. In the absence of phosphatase inhibitors no 14-3-3 binding was observed in the overlay assay (not shown). Thus, in the overlay assay 14-3-3 interacts primarily with phosphorylated target proteins in Hydra extracts.

Comparison of the overlay assays performed with DIG-14-3-3 Hy A, DIG-14-3-3 Hy B, and DIG-BMH 1 probes revealed that the Hydra 14-3-3 probes react much stronger with 14-3-3 than from hydra lysates than with 14-3-3 target proteins (Fig. 6b). It is possible that the DIG label which is covalently bound to free amino groups (e.g., ε amino groups of lysine residues) has an impact on the binding affinity of the probe. Interestingly, 14-3-3 Hy A has seven and 14-3-3 Hy B has five lysine residues more in the amino acid chain than BMH 1. Thus, the label might inflict steric hindrance for binding of the Hydra probes to phosphorylated target proteins but not for dimerisation. DIG-BMH 1, on the other hand, does not recognise 14-3-3 proteins in Hydra lysates in overlay assays very well (Fig. 7). Only after overexposing the blot shown in Fig. 7 two weak bands at 32 and 33.5 kDa could be seen. These bands were not blocked by the phosphopeptide and therefore most likely represent Hydra 14-3-3 proteins (not shown). This could be due to impaired heterodimerisation between 14-3-3 isoforms of different species.

14-3-3 proteins in Hydra are localised in the cytoplasm of most cells, in the nucleus of epithelial cells, and in food granules of endodermal cells

Probing macerates with anti 14-3-3 antibody

To determine the subcellular localisation of 14-3-3 protein in Hydra we stained Hydra whole mounts and macerated cells with the K19 antibody. The results were similar and are summarised in Fig. 8. Epithelial cells were stained throughout the cytoplasm (Fig. 8a and b). Weak staining of the nucleus could also be observed, albeit not in every nucleus (Fig. 8b). In endodermal epithelial cells, strong 14-3-3 staining was observed in the food granules (Fig. 8c, e, and f). Food granules can be detected in phase contrast as dense granules (Fig. 8d). For unknown reasons they stain with all DNA dyes we have used so far (Fig. 8g, TO-PRO, red). They also stained brightly for 14-3-3 (Fig. 8f, green). When all three panels were compared, it became clear that 14-3-3 and TO-PRO stained many of the same granules, but with different intensities (Fig. 8f, g, and merged image e). This demonstrates differential contents in such granules. In long-term starved animals the number of food granules declined. The food granules that were present, however, were again TO-PRO- and 14-3-3-positive (not shown). While 14-3-3 staining in epithelial cells was mostly in the cytoplasm, we also observed nuclear staining in some cells. The number of stained nuclei increased when the animals were starved. In macerates from sexual polyps many epithezial cell nuclei were stained with 14-3-3 antibody (Fig. 8b). Such hydra (AEP) had been induced to develop oocytes and sperm cells by a strict change in their feeding regime from heavy feeding (daily) to very sparse feeding (once a week).

Interstitial cells and their differentiated products, nematocytes, and nerve cells (Fig. 8h, j, and l, phase contrast), revealed very strong cytoplasmic staining that showed a granular pattern (Fig. 8i, k, and m). Nuclear staining was not observed in cells from the interstitial cell lineage.

Probing macerates with DIG-labelled 14-3-3 probes

DIG-labelled 14-3-3 Hy A and Hy B interacted strongly with 14-3-3 proteins on Western blots. Therefore we used these probes as alternative reagents to the K19 antibody to probe macerates. Both DIG-labelled Hydra 14-3-3 isoforms could interact with 14-3-3 on macerates. The signal obtained with DIG14-3-3 Hy A was stronger than the signal with DIG-14-3-3 Hy B.

Overall, DIG-14-3-3 Hy A stained the same structures as K19 antibody. Cytoplasmic staining in a granular pattern could be found in nematocytes (Fig. 9a and b) while in epithelial cells the cytoplasm was stained more uniformly (Fig. 9c and d). The food granules in endodermal cells were also stained. In both ectodermal and endodermal epithelial cells the nuclei were stained (Fig. 9c and d and e and f). Surprisingly, the nuclear signal was much stronger with DIG-14-3-3 Hy A than with K19. It was present in all epithelial cells but absent from interstitial cells and their differentiated products.

Discussion

14-3-3 proteins are highly conserved and very abundant proteins present in all eukaryotes. In higher animals and plants multiple isoforms of 14-3-3 proteins are present. By binding to target proteins in a phosphorylation-dependent manner 14-3-3 proteins influence their activity and thus provide a means for posttranslational regulation of signalling cascades.

In lower animals, like Drosophila and Caenorhabditis, and in yeast only two isoforms have been described so far. We have cloned two 14-3-3 isoforms from Hydra and found transcripts of both isoforms in all cell types and body regions of Hydra. 14-3-3 Hy B appears to be expressed in the endoderm at a slightly higher level than in the ectoderm.

It has been reported that 14-3-3 isoforms from mammals and from plants can form homo- and heterodimers. Heterodimers also occur in vivo and are thought to provide the basis for the diversity of cellular functions in which 14-3-3 proteins can be involved [19]. We have shown in Far Western overlay assays that the two isoforms of Hydra 14-3-3 also can form homo- and heterodimers. 14-3-3 Hy A interacts with itself. It also interacts with different isoforms in cell lysates. 14-3-3 Hy B, on the other hand, only bound one
isoform in cellular lysates and interacted only with 14-3-3 Hy A expressed in *E. coli*. Thus it appears that 14-3-3 Hy B does not form homodimers in the overlay assay. Under more native conditions in the yeast two hybrid system 14-3-3 Hy B could form homodimers (data not shown).

Probing Western blots with the anti 14-3-3 antibody K19 and with DIG-labelled 14-3-3 Hy A, we always found three bands. At this point we cannot be sure that the two 14-3-3 isoforms we have cloned from *Hydra* are the only two isoforms that are present or if the third band represents a third isoform.

14-3-3 protein is found in all cell types. The cell separation experiment shown in Fig. 3 suggests that the isoforms are differentially expressed in different cell types. This interpretation is hard to prove at the moment as we do not have reagents which distinguish between both *Hydra* 14-3-3 isoforms in immunocytochemistry. We cannot rule out the possibility that the cell separation procedure, which included prolonged incubation of the cells with pronase, could have had an effect on protein stability and/or expression. Cell-type-specific expression of mRNA is, however, observed in elutriated cells [20].

Subcellular distribution of *Hydra* 14-3-3 is mainly cytoplasmic but also nuclear. Nuclear localisation of 14-3-3 proteins has been shown immunocytochemically in plants [21] and recently in the parasite *Schistosoma mansoni* [22]. It has also been suggested that 14-3-3 which is not bound to target proteins localises to the nucleus [23]. This interpretation could explain why we only detect weak nuclear signals with the anti 14-3-3 antibody in immunocytochemistry while the DIG-labelled probes bind strongly to the nucleus on macerates. Their interaction with 14-3-3 depends on dimerisation. In our overlay assay they can easily interact with 14-3-3 proteins that have been denatured and treated with reducing agents and are therefore monomeric. On macerates, however, 14-3-3 proteins could still be dimers after fixation of the cells with paraformaldehyde. The probe would then probably interact better with monomers that could be present when no targets are bound to the 14-3-3 protein. Interestingly, in *Hydra* the nuclear distribution is associated with starvation, which becomes particularly obvious in sexual polyps where starvation causes the whole reproduction mode to change from vegetative to sexual. This hints towards the possibility that 14-3-3 in *Hydra* could confer a regulatory role on proteins that are involved in metabolic regulation. A second clue comes from the strong staining of food granules by the anti 14-3-3 antibody. Food

and in food granules of an endodermal cell (e) indicate colocalisation of the DNA dye TO-PRO with 14-3-3 protein. This colocalisation becomes clearer in single sections which are provided for the epithelial cell nucleus of sexual hydra (AEP strain) in (b). The top panel shows the merged images of the DNA stain (middle panel, red) and the 14-3-3 stain (lower panel, green). Single sections that show food granules in an endodermal cell are shown in f (14-3-3, green) and g (TO-PRO, red). Scale bars, 10 μm.

Fig. 8. Confocal images of hydra cells stained with the anti 14-3-3 antibody K19. Ectodermal cells (a) endodermal cells (c–g), interstitial cells (h, i), nematocytes with differentiating capsules (j, k) and nerve cell (l, m) are depicted. Phase-contrast images are provided for comparison of the cellular structures (d, h, and l). (a, c, e, i, k and m) Merged images of projections obtained in the green channel (14-3-3 signal) and in the red channel (TO-PRO signal). The yellow colour in those images that can be seen in the nuclei of epithelial cells (a and c), on the edge of the nerve cell nucleus (m),
Granules are obviously not only present or stained by anti-14-3-3 antibody immediately after feeding. Some of them last for at least 2 weeks of starvation. All this time they seem to contain 14-3-3 protein. 14-3-3 protein has previously been shown to be involved in metabolic regulation in plants. It appears to modify the stability of enzymes involved in sugar metabolism [2,4,24] and has been implicated in regulation of starch composition and accumulation. It is specifically localised in starch granules [24]. It is tempting to speculate that 14-3-3 performs a similar metabolic function in food granules in Hydra, possibly by regulating enzymes involved in storing nutritional compounds.

From our protein localisation studies it appears that Hydra 14-3-3 proteins could have very diverse functions and bind target proteins not only in the cytoplasm, but also in the nucleus. We have identified five prominent target proteins for 14-3-3 proteins in Hydra with molecular weights between 50 and 120 kDa. The binding was phosphorylation-dependent and could be inhibited by competition with a phosphorylated 14-3-3 consensus peptide for the binding site. We are now trying to purify these proteins on a 14-3-3 affinity column in order to establish their molecular identity. This should provide a more complete picture of the role played by 14-3-3 proteins in Hydra.

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Fig. 9. Macerates were probed with DIG-14-3-3 Hy A and stained with FITC-anti-DIG antibody. (a and b) Nest of differentiating nematocytes. (a) Merged image of projections of DNA-stained and DIG-14-3-3-stained images. (b) DIG-14-3-3-stained image, green. (c and d) Endodermal epithelial cell. N, nucleus; G, food granule. Compare again merged images from DNA and DIG-14-3-3 stain (c) with DIG-14-3-3 stain alone (d, green). Staining with DIG-14-3-3 in granules and in the nucleus is weak, but both structures are recognisable in d and yellow colour in c indicates colocalisation of DNA dye and 14-3-3 protein. (e and f) An ectodermal epithelial cell with staining in the cytoplasm and in the nucleus (merged images TO-PRO and DIG-14-3-3 in e; DIG-14-3-3 in f). Scale bars, 10 μm.
References


