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Original Article

Patterning a multi-headed mutant in *Hydractinia:* enhancement of head formation and its phenotypic normalization

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ABSTRACT In a mutant strain of *Hydractinia* (Cnidaria: Hydrozoa), the polyps develop ectopic supernumerary tentacles and heads (hypostomes) after an initial phase of wild-type growth. In order to elucidate the molecular mechanisms implicated in the development of aberrant phenotypes, we tried to enhance or suppress the expressivity of this hypomorphic mutation by exposing subclones to factors supposedly influencing pattern formation. Upon iterated treatment with alsterpaullone, an inhibitor of GSK-3, the formation of additional, ectopic head structures and the budding of new polyps were dramatically accelerated and enhanced. The endogenous stolon-inducing factor (SIF) had opposite effects by reducing head forming potential while increasing stolon-forming potential. SIF could be used to rescue extremely aberrant phenotypes. In these mutant colonies, long polyps with multiple heads eventually detach from stolons and lose the ability to regenerate stolons. Upon exposure to SIF, such free-floating multi-headed polyps resumed production of stolons and acquired wild-type morphology. We conclude that a canonical WNT signaling cascade is involved in patterning the body axis of polyps and in the initiation of budding, and that SIF counteracts this signaling system.

KEY WORDS: *Hydractinia, Cnidaria, pattern formation, GSK-3, alsterpaullone, SIF*

Introduction

The availability of mutants is one of the features fostering the choice of *Hydractinia echinata* (Cnidaria: Hydrozoa) as a cnidarian model organism in studies on the evolution of genes, developmental mechanisms, and allorecognition (Frank *et al.*, 2001). *Hydractinia* is a colonial species; the colony consists of hydra-like feeding polyps (gastrozooids) and sexual polyps (gonozooids); these polyps are all interconnected with each other through a network of gastro-vascular channels, termed stolons. This gastro-vascular network serves in the distribution of nutrients, regulatory signals and migratory cell types.

Among the mutant phenotypes available there are several clones in which the polyps or stolons display abnormal traits (Müller, 2002). In one category of such mutants the feeding polyps develop multiple heads with time. In all these clones the mutation is hypomorphic: Young polyps arising from metamorphosing larvae and, in the post-metamorphic life phase, arising from buds on stolons, initially display a normal phenotype. However, upon attaining the size of full-grown wild-type polyps and fed with lipid-rich *Artemia* nauplii, they continue to grow and elongate, forming additional heads in more or less regular distances along the body column. Eventually, the multi-headed polyps resorb the stolon tissue at their base and detach from the substratum. Such free-floating multi-headed forms are unable to adhere to any substratum and to reproduce. Thus, two antagonistic traits characterize the mutant: An age-dependent increase of the head-forming potential is associated with a concomitant loss of the ability to form stolons.

In order to elucidate possible molecular mechanisms underlying this mutant phenotype, we tried to influence the expressivity of the mutation in antagonistic directions: To speed up and enhance multi-head formation on the one hand, and conversely, to rescue detached multi-headed polyps.

Enhancing head formation

Strong, though indirect, evidence points to a pivotal role of a canonical WNT signaling cascade in initiating head formation in

Abbreviations used in this paper: GSK, glycogen synthase kinase; PKC, protein kinase C; SIF, stolon-inducing factor.

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Hydra (Hobmayer, *et al.,* 2000). Since soluble WNT molecules are not yet available, we tried to activate downstream events of the WNT pathway by treating colonies with pharmaceutical inhibitors of the glycogen synthase kinase GSK-3. Inhibition of GSK-3 is expected to allow accumulation of stabilized β-catenin in the cytoplasm and its translocation into the nucleus, where it eventually activates transcription factors of the Tcf/Lef family (review: e.g. Wodarz and Nusse, 1998).

Rescuing the lost ability to form stolons

Aiming at rescuing detached multi-headed feeding polyps, we exposed them to SIF, an endogenous stolon-inducing factor. The development of the network of stolons resembles the development of the network of blood capillaries (angiogenesis) in vertebrates in several aspects: The stolon tip is a motile, pathfinding organ, paralleling the function of the terminal cell at the tips of capillaries. The tips of neighboring stolons attract each other, and upon encounter, they fuse forming a continuous vascular lumen. The chemical signal that is released by these tips to mutually attract each other is SIF. It was designated SIF because of another function: When an advancing stolon tip approaches an established stolon in its path of growth, the SIF produced at this tip induces the formation of a lateral tip along the flank of the encountered stolon. The induced tip gives rise to a branch (Lange and Müller, 1991) that grows towards the source of SIF, seeking contact with the emitter of the signal, and eventually fuses with it. (This branch-inducing capability parallels the action of angiogenic factors in vertebrates; reviewed, e.g., in: Roman and Weinstein, 2000).

SIF not only is able to induce branching of stolons; under experimental conditions it also induces transformation of polyps into stolons (Lange and Müller, 1991). Therefore, we expected an influence of SIF on the phenotype of multi-headed polyps. However, since the chemical composition of SIF was not known when this study was begun, and SIF preparations were not available, prerequisites to the present experiments were the collection of SIF released by many thousands of primary polyps, and the purification of the effective substance. The purification procedure in addition provided hints as to the chemical nature of SIF.

Results

Alsterpaullone treatment: acceleration and enhancement of multi-head formation

Preliminary experiments with embryos and larvae of *Hydractinia* led us to select alsterpaullone as the most potent GSK-3β inhibitor available at present. Alsterpaullone competes with ATP for binding to GSK-3β (Leost *et al.,* 2000). For the present experiments eight week old subclones of the hypomorphic, slowly growing clone *mh* 7x7-21 were used. Very young feeding polyps of this clone display a normal appearance, larger polyps form single ectopic tentacles below the tentacle crown. Ectopic hypostomes are only formed after several months of colony growth in long, well-fed polyps. At the beginning of the experiment, a third of the polyps had developed one or a few ectopic tentacles, but no ectopic heads (hypostomes with a mouth opening) were present.

Eight experimental colonies, comprising initially a total of 149 feeding polyps, were daily subjected to a pulse-type treatment with a low dose (500 nM) of alsterpaullone at 7 successive days (see Methods). Twelve colonies of the same age comprising 156 polyps initially were incubated with 1ppm DMSO and served as controls. In addition, some subclones of the wild-type clone male1 comprising several hundreds of polyps were subjected to the same regime of treatment.

Formation of ectopic head structures

At the chosen low concentration and short incubation time no immediate short-term effects of the alsterpaullone treatment were apparent either in the wild-type or in the mutant colonies. Feeding behavior and growth were not impaired. However with time, the polyps of the alsterpaullone-treated *mh*7x7-21 colonies formed more and more ectopic tentacles and eventually even hypostomes. In the first 5 days of the treatment period the number of supernumerary tentacles per polyp increased only moderately. But

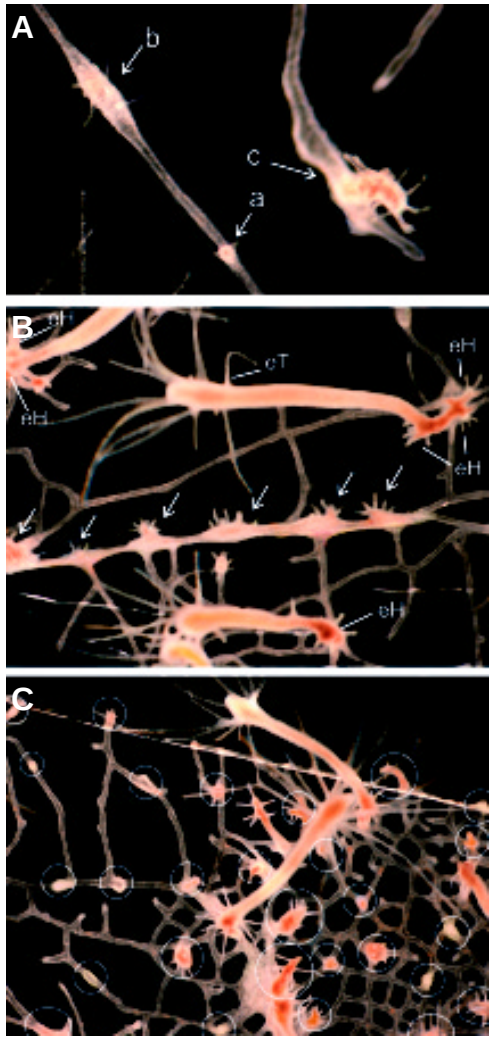

Fig.1. (A-C) Stimulation of ectopic head formation and budding by alsterpaullone in young colonies of the clone *mh*7x7-21. (A) *Arrow "a" points to a normal-sized bud, arrow "b" to an enlarged bud forming tentacles earlier than normally and arrow "c" to a young polyp emerging from an enlarged bud and studded with (ectopic) tentacles.* **(B)** *Characteristic phenotypic aberrations: eT, ectopic tentacle; eH, ectopic hypostomes; arrows point to polyps arising from a swollen stolon.* **(C)** *High spatial density of arising new polyps. Buds which appeared between day 6 to 9 of the treatment period (see Fig. 2) are encircled.*

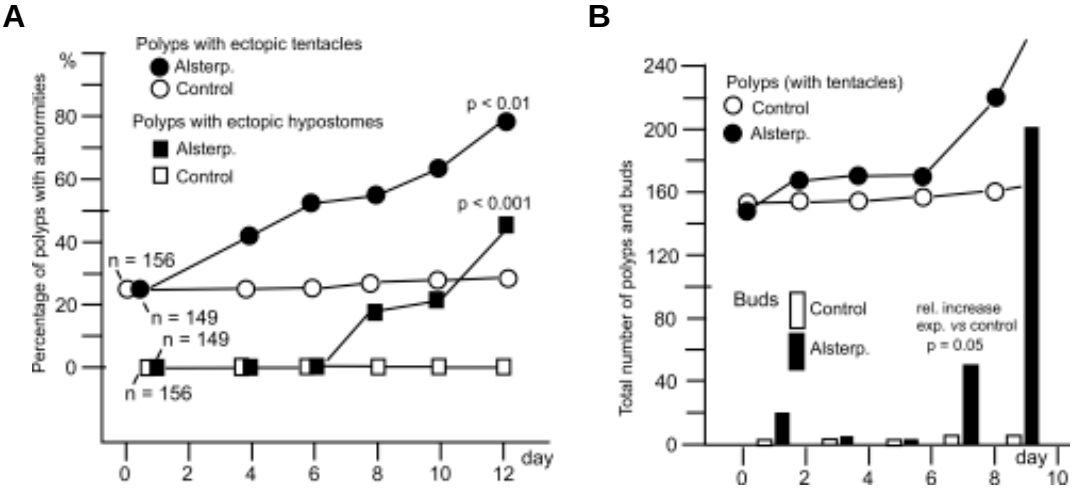

Fig. 2. Time course in the occurrence of ectopic head structures and budding activity in alsterpaullone-treated colonies. **(A)** *Ectopic tentacles and heads (hypostomes) in mutant colonies exposed to alsterpaullone (filled symbols) and in DMSO-treated control colonies (open symbols). Experimental and control colonies were subclones of mh7x7-21. Treatment was stopped at day 8, values were counted up to day 12. Since the colonies bud new polyps almost daily and buds initially lack tentacles, the counted values were related to the number of polyps having formed tentacles at the time point of counting.* **(B)** *Budding activity. Total number of feeding polyps (with tentacles) and number of newly generated buds (still without tentacles) are compared during the experimental period. Statistics: analysis of contingency tables using the chi-square test in the version of Fisher´s exact test.*

between day six and eight a sudden appearance of ectopic head structures occurred, documenting an increase of the head forming potential. Young polyps arising from the buds soon formed ectopic tentacles and became studded with tentacles all over their body column (Fig. 1A). Older, merely slowly growing polyps exhibited a delayed but similar response. They formed some additional ectopic tentacles in the upper gastric region but also complete ectopic heads with a mouth opening, most frequently near the base of the body column. Examples of such polyps are shown in Fig. 1B,C; quantitative data are summarized in Fig. 2.

Male1 wild-type polyps exposed to alsterpaullone, as well as the DMSO-treated controls of both the *mh7*x7-21 and male1 colonies, remained unchanged during the two week period of the experiment and the following six weeks of further observation.

Detachment of multi-headed polyps

Several polyps of the alsterpaullone-treated mutant colonies detached from the stolons and formed a head at their lower end like the multi-headed polyps of untreated, ageing colonies of this clone. In addition, a new type of separation was observed: large ectopic heads splitting into two (Fig. 3A), and the body column separating between the two heads (Fig. 3B). Eventually, such detached polyps gave rise to free-floating and branching multi-headed tubes (Fig. 3C) like those produced by ageing colonies of this clone (Fig. 4A).

Budding activity and enlarged buds

In the alsterpaullone-treated mutant colonies, polyp buds emerged from stolons at high frequency (Fig. 2) and short distances from each other and existing polyps (Fig. 1 B,C). In addition, an overall increase of the potential to form head structures caused responses in the stolonal compartment never previously documented. Enlarged buds and also some buds of almost normal size formed tentacles even before the body column arose (Fig. 1A). At several locations in the colonies stolons swelled up over some distance and formed a series of tentacles. Subsequently hypostomes were inserted between bundles of tentacles, and eventually gave rise to polyps (Fig. 1B, arrows). Such blown-up stretches stolons forming polyps at unusually short distances can be interpreted as one spatially extended bud which then splits up into several buds, thus displaying a behavior known from experimentally enlarged morphogenetic fields.

Frequently, the stolon tissue between buds located at close distances retracted and the stolons became fragmented. Normally, buds incorporate stolon tissue moving towards evaginating and elongating buds (Müller, 1964) as do buds of *Hydra*. The current fragmentation of the stolons observed may be due to depletion of cell material in between the buds but partially also reflects cell death (current studies).

In morphological as well as molecular terms bud formation can be considered as natural additional, ‘ectopic’ head formation.

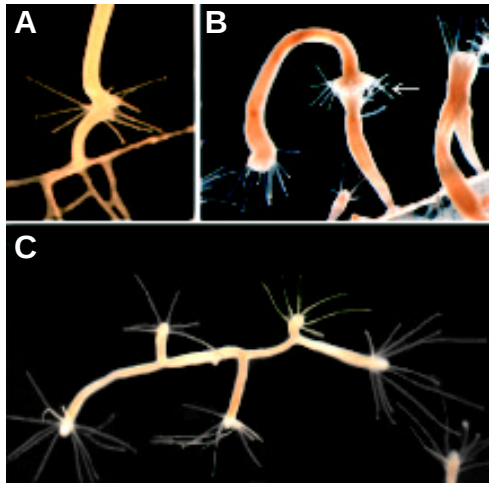

Fig. 3. Separation of multi-headed polyps. **(A)** *Polyps with split ectopic heads.* **(B)** *With time, the body column separates in between the two heads; this separation results in a free-floating polyp and one polyp remaining in contact with the stolon.* **(C)** *Detached polyps with a hypostome at each end and three additional hyopostomes between the terminal heads.*

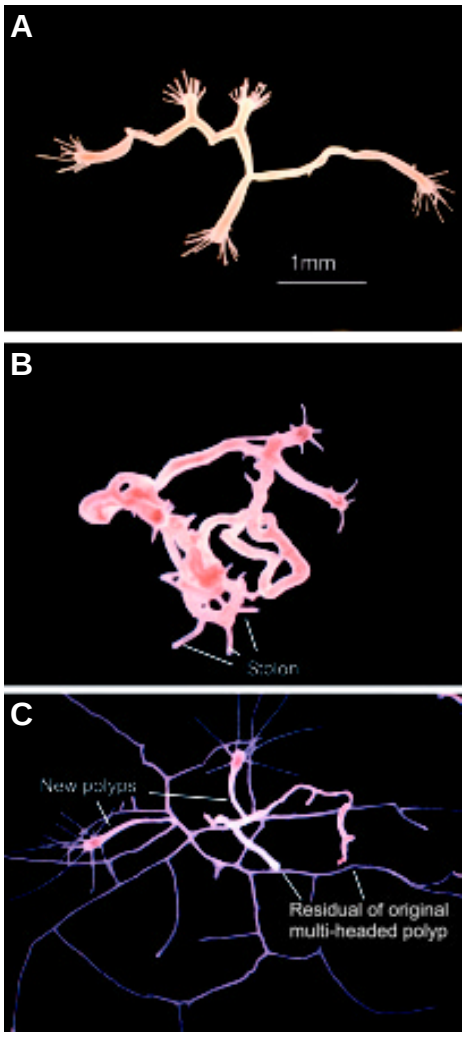

Fig. 4. Induction of stolon formation by SIF in a stolon-less, multi-headed mutant. *(A-C) Temporal sequence.* **(A)** *Example of a multi-headed, free-floating specimen which has separated from a one year old, ageing stock colony.* **(B)** *Onset of stolon formation; the heads are in the phase of regression. The multi-headed forms were exposed to SIF produced by about 100,000 primary polyps and purified by chromatography as shown in Fig. 6. As more than half of the active fractions had been invested in bioassays in order to determine the position and dimension of activity peak, the residual activity left for this experiment corresponded to SIF produced by about 30,000 polyps.* **(C)** *From the original specimen, only a residual, head-less stalk is left; a stolon net has formed and the stolons bud new polyps with (initially) wild-type morphology.*

Thus, the effects of low doses of alsterpaullone can be summarized as general increase of the potential to form head structures.

In previous, preliminary experiments, higher doses of alsterpaullone and longer incubation periods provoked ectopic head formation much earlier (data not shown), but with severe after-effects. Within two weeks all experimental polyps lost the ability to engulf food, and subsequently regressed. Stolonal growth continued for several days at the expense of the resorbed polyps, but these stolons did not bud new polyps. Eventually, the residual tissue disintegrated.

Rescue of a multi-headed mutant by exposure to SIF

Nine multi-headed forms of the clone *mh7*x7-21, including the example shown in Fig. 4A, and comprising 33 heads were transferred to a dish of which the bottom was covered with a layer of SIF molecules. The amount of SIF invested was the product of about 30 000 primary polyps and the nominal concentration chosen was that which was sufficient to induce stolon formation in the gastric region of primary polyps settling and attached onto such SIF-covered substrata (see Materials and Methods). Another 12 of such multi-headed forms with a total of 42 heads served as untreated controls. These control specimens did not change their morphology during the following six months, unlike the specimens that came in contact with the SIF-covered substratum. All the multi-headed polyps resorbed their heads and began to sprout stolons. The stolons attached to the bottom of the dish, branched and formed a stolonal network with normal morphology (Fig. 4 B,C) as do young colonies of this mutant clone. The stolons budded new polyps with one single terminal head (hypostome), while the original multi-headed structures gradually disappeared. The colonies acquired a wild-type morphology and retained this apparently normal phenotype for several weeks.

SIF was applied only once. Since the animals adhere to the substratum as does SIF (for removal of SIF, solvents such as methanol or DMSO have to be used), the time of incubation could not be restricted to a predefined period. As the experiments were not conducted under strict sterile conditions we assume that SIF activity vanished within a few days or even hours. If so, the SIF effect is long lasting. However, as the treatment with SIF did not

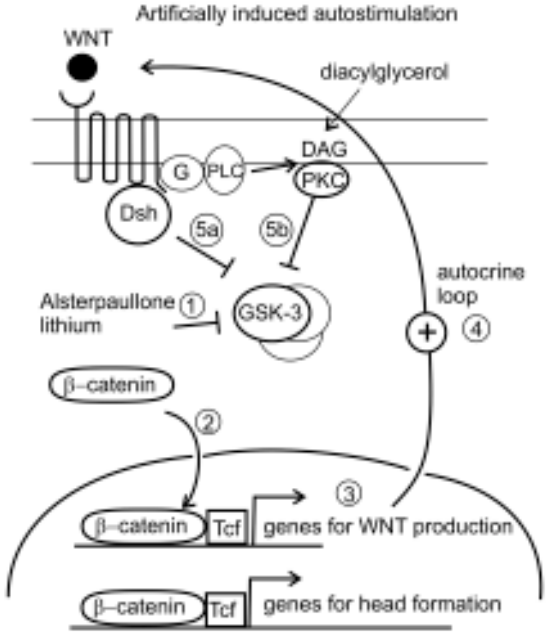

Fig. 5. Proposed positive feed back loop in a canonical WNT pathway. *The figure includes a proposal of how stimulation of (ectopic) head formation through activation of PKC (Müller, 1989) could be integrated in this system in a simple, straightforward manner. This last proposal refers to a report by Wang and Malbon (2003) summarizing evidence that in several instances, a ligand-activated FRIZZLED receptor stimulates PKC activity via G protein and phospholipase C.*

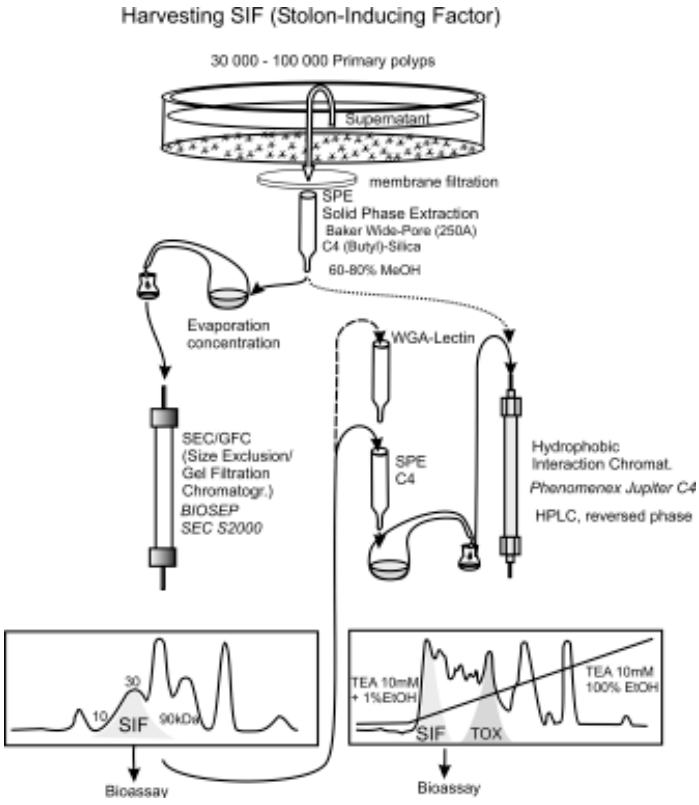

Fig. 6. Flow diagram of the methods used to collect and purify SIF. *Dashed lines indicate routes taken occasionally to characterize the activity (WGA column) or to shorten the procedure to collect sufficient amounts of activity for the experiment shown in Fig. 4.*

change the genotype but merely reflected the phenotypic plasticity of the hypomorphic mutation, some of the polyps eventually became multi-headed after 2-6 months. A lasting rescue of those mutants would hence require iterated treatments with SIF.

The procedures used to collect, purify and inactivate SIF characterize it as a small glycoprotein with a MW of about 10 kDa but a strong tendency to form aggregates. Inactivation by proteinase K, binding to WGA, permeation through ultrafilters and size-exclusion chromatography (for details see Materials and Methods) provided the arguments for this conclusion. In a previous study (Lange and Müller, 1991) SIF was resistant to cleavage by trypsin, and therefore its peptidic nature remained covert until proteinase K was applied to attack SIF. Proteinase K hydrolyses polypeptides at the carboxy group of aliphatic, aromatic and hydrophobic amino acids. This cleavage pattern may provide useful hints for future efforts to determine the amino acid sequence of SIF.

Discussion

Organizing centers

Like in most multicellular organism, in hydroids axis formation and patterning of the body axis is controlled by two opposite organizing centers, the head organizer and the foot or stolon organizer (*Hydra:* Broun and Bode, 2002; Bode, 2003, and references therein). Both centers interact as has been shown in *Hydra* (Müller, 1990, 1995, 1996). This interaction does not imply stringent mutual dependence. Polyps of *Hydra,* as well as of *Hydractinia,* can be caused to entirely transform into head structures by iterated treatment with activators of PKC such as membrane-permeable diacylglycerols or tumor-promoting phorbolesters (Müller, 1985, 1989, 1990).

On the other hand, *Hydractinia* polyps can also be completely transformed into stolon tissue by various kinds of treatment, for instance by exposure to high doses of SIF (Lange and Müller, 1991).

The results presented here point to an insufficiently balanced production of morphogens supporting head or stolon formation, respectively, in the mutant clone. The balance can experimentally be shifted toward an increase in the head forming potential by alsterpaullone, toward an increase in the potential to form stolons by treatment with SIF.

The GSK-3 and WNT system

Assuming that axis formation and positional value are under the control of a canonical WNT system (Hobmayer *et al.,* 2000), the action of alsterpaullone can tentatively be attributed to its ability to inhibit GSK-3α/β (Glycogen Synthase Kinase-3α/β). This inhibition would result in activation of the transcription factor Tcf by stabilized β-catenin (Wodarz and Nusse, 1999; Leost *et al.,* 2000, and references therein).

However, following current models, alsterpaullone would lead to an activation of downstream β-catenin/Tcf target genes, but not to the installation of an upstream WNT-signaling center at the top of the cascade. In *Hydra* in the center of buds, and likewise at the top of decapitated body columns and in the center of body axes emerging from aggregates, WNT-expressing head organizers are established (Hobmayer *et al.,* 2000). In the mutant analyzed here, the significant effect of alsterpaullone is induction of budding and ectopic head formation. Stimulation of downstream events in the WNT cascade by alsterpaullone eventually results in the establishment of new WNT-expressing spots, that is of new starting points of the cascade, along the body column of polyps and along stolons (current studies). We propose to supplement the canonical model by a positive feedback loop: Activation of β-catenin/Tcf-controlled genes would in turn lead to the production and secretion of WNT (Fig. 5).

A positive, reinforcing feedback Tcf/Wnt loop has also been implicated with endomesoderm specification in the sea urchin embryo (Davidson *et al*., 2003, and references therein) and head regeneration in *Hydra* (Holstein *et al*., 2003). When a head is removed, the source of WNT is removed and must be re-established. The distribution of nuclear β-catenin and Tcf along the body column might be components of the positional value which enables re-establishment of a source of WNT signaling molecules. Induction of ectopic head formation by activators of PKC (Müller, 1985, 1989) can be included in the model of a positive feedback loop in a simple, straightforward manner (Fig. 5).

While this study was carried out, a report communicated at a meeting documented that in *Hydra* alsterpaullone also induces the establishment of head organizers and the sprouting of ectopic tentacles (Broun *et al*., 2003).

To evoke ectopic head formation in clones of *Hydractinia* with alsterpaullone, an optimum dose and incubation period must be found. Supra-optimal doses caused regression of heads, and the stolons lost the ability to bud new polyps. Apparently, over-optimal

doses suppress the capability to establish new head-organizing centers. Alsterpaullone is also a potent inhibitor of several cyclin-dependent kinases (Schultz *et al.,* 1999; Leost *et al.,* 2000), and blockage of the cell cycle in stem cells and dividing derivatives might well contribute to the adverse after-effects observed here.

Another known inhibitor of GSK-3, lithium, evoked similar antagonistic responses in *Hydra vulgaris*. A particular pulse-type treatment induced the development of oversized heads and supernumerary tentacles, while long-term exposure to lithium caused the development of ectopic feet and suppressed the capacity to regenerate heads (Hassel and Berking, 1990; Hassel and Bieller, 1996).

Antagonism by the stolon-inducing factor SIF

Compared to the artificial drug alsterpaullone, the natural factor SIF had an antagonistic effect in polyps; it caused lowering of positional value, that is a decrease of the potential for head formation combined with an increase of the potential for stolon formation. This effect was not anticipated when SIF was detected. In the first instance SIF is a factor resembling angiogenic factors in inducing sprouting, branching and interdigitation of vessels in the formation of a vascular network. Unexpectedly, SIF additionally has the capability of counteracting head-promoting morphogens in polyps. Induced by SIF, heads of metamorphosing polyps transform into a stolon tip (Lange and Müller, 1991) and, as has been shown here, multi-headed, free floating polyps resume stolon formation. Perhaps SIF has additional roles in pattern formation and differentiation, in analogy to the multiple roles angiogenic factors have in vertebrate embryos (review: Roman and Weinstein, 2000).

Materials and Methods

Raising and subcloning of Hydractinia

Hydractinia is routinely raised in our lab (Frank *et al.,* 2001; Müller, 2002; www.zoo.uni-heidelberg.de/frank/hydractinia). Subclones of the strains used in this study were raised from explants. Pieces of the stolon plate bearing several feeding polyps were cut out and carefully removed from the substratum together with the chitin-containing basement layer. The pieces included a part of the central incrusted area of the stolon plate (for better handling them) and a part of the soft peripheral area (for better growth). After transfer to their new location the pieces were held in place with glass splinters or glass pearls until the regenerating tissue resumed growth and adhered to the substratum with newly secreted adhesive basement layer.

The animals were fed with *Artemia* nauplii every second day.

Treatment with GSK3-inhibitors

GSK-3 inhibitors were obtained from CALBIOCHEM. Stock solutions of 5mM alsterpaullone (9-Nitro-7,12-dihydroindolo[3,2-d][1]benzazepin-6(5H)-one) were prepared with DMSO. This stock solution was first diluted with DMSO to obtain 0.5, 0.25 and 0.125 mM in DMSO. For treatment of the animals, 1µl of the DMSO solutions was added per ml of seawater, to obtain final working solutions containing 0.5, 0.25 or 0.125 µM alsterpaullone and 1 ppm DMSO. The data presented here are from colonies treated daily with 0.5 µM alsterpaullone for 4-5 hrs. The control colonies were treated with 1 ppm DMSO only; thus the final concentration of DMSO in all experimental and control dishes was kept at a constant 1 ppm.

Harvesting and concentrating SIF

The stolon-inducing factor SIF is released in trace amounts into the medium by primary polyps and young colonies during the phase of stolon formation. Besides SIF, the animals also release toxic components by discharging a particular type of nematocysts to eliminate neighboring competitors of the living space (Lange *et al.,* 1989). Therefore, we first had to develop methods to enrich and purify SIF, guided by bioassays. In addition, these procedures were designed to collect more information on its chemical nature.

For each single experiment that included a chromatographic step, about 30,000 planula larvae had to be collected. Batches were stored at 6°C until the amount of 30,000 was assembled. The larvae were transferred into glass bowls and induced to enter metamorphosis by incubating them in seawater enriched with 56 mM Cs^+ for 3-4 h at RT. After having settled on the bottom of the bowls, the attached metamorphosing larvae were washed several times. At 18ºC the larvae complete metamorphosis in 24 h.

The resulting primary polyps release traces of SIF into the surrounding seawater. The conditioned water was removed 48-62 h after induction of metamorphosis, sterilized by membrane filtration and slowly sucked through previously activated SPE columns (Baker, Wide-Pore (250-300A) C4-(butyl-)silica columns). The bound biological activity was eluted with 70% MeOH. SIF tolerates evaporation of the solute to dryness under reduced pressure and cooling. Pooled methanolic or dried samples were stored at –20°C.

Bioassay of SIF

The bioassay was standardized as follows. Dried samples were re-dissolved with methanol. Increasing amounts of the solution were poured in succeeding 2.9 ml wells of polystyrol cell culture plates. The MeOH was completely evaporated under a stream of nitrogen, or by lyophilization. Then 200 µl of seawater containing about 20 metamorphosing larvae were added to each well. The primary polyps develop characteristic SIF-symptoms within a week: The base of the polyps does not sprout stolons, instead the stolon-forming position is shifted up into the gastric region; at high concentrations the entire polyps transform into stolon tissue and their head is replaced by a stolon tip.

Quantification of SIF was based on the comparison of dose-response curves established in the same way. After enzymatic digestion, or after chromatography, SIF was re-collected with SPE columns and the amount of activity determined by comparing dose-response curves before and after the chosen analytical or preparative step. Loss of activity causes a shift of the curves to the right: higher amounts of methanolic SPE eluates were needed to reach the $D_{50.}$ Progressing purification was indicated by a shift of the curves to the left: lesser amount of eluates was needed to cause 50% of the animals to develop SIF symptoms.

Characterization and chromatographic purification of SIF

Enzymatic inactivation of SPE elutes was tested using several enzymes that cleave peptide bonds. The biological activity was destroyed after passing SIF preparations through columns with proteinase K bound to a matrix (F7m CR-columns, MoBiTec).

Affinity chromatography with lectin WGA (wheat germ agglutinin) was performed using affinity columns (Sepharose-WGA, Sigma). They were eluted with 1M N-acetyl-glucosamine. Before performing bioassays, N-acetyl-glucosamine was removed by ultrafiltration using filters with 3kDa exclusion limit. The supernatant was further purified using SPE columns. Recovery was 33 to 50% compared to an aliquot of the SIF preparation not subjected to WGA affinity purification.

Enzymatic degradation of the activity by proteinase K and its ability to bind to WGA lectin prompted us to select procedures appropriate to extract and purify glycoproteins. The pooled samples were subjected to several procedures of chromatography (Fig. 6) using volatile eluents.

SEC (Size Exclusion Chromatography, Gel filtration) was performed using a Pharmacia Sephacryl S-1000 column (16/60) equilibrated and eluted with 20% MeOH, or a Phenomenex BioSep-SEC S-2000 column (300 x 7.5), equilibrated and eluted with high salt solutions to reduce the formation of SIF aggregates. Each second fraction was tested for biological activity. As in chromatographic fractions activity increases up to a peak and then decreases, the result of bioassays were dose-response curves with increasing and decreasing SIF concentrations.

Despite eluting with methanolic solutions or high salt solutions of various composition the biological activity was always distributed among a quite wide range of fractions. According to the position of the peak activity, the molecular mass of SIF calculates to about 30 kDa. At the lower end of the active range the calculated molecular mass was ≤ 12 kDa, at the upper end it was 90 kDa. Re-chromatography of pooled fractions with SIF activity exhibiting apparent molecular masses >36 kDa, and those having <36 kDa, yielded the same distribution ranging from 12 to 90 kDa. This indicates that SIF tends to form aggregates with varying multiplication factors.

Ultrafiltration (Amicon Centricon) pointed to a molecular weight of ≥10 kDa, as 96% of the SIF activity were retained on a membrane with a nominal exclusion limit at 10 kDa, and residual 3% were gathered on a subsequent 3 kDa cut-off membrane.

Hydrophobic interaction was performed using a Phenomenex Jupiter HPLC column (C4, 5 μm, 250 x 4.6). For elution, a gradient was run with starting buffer A (10mM TEA + 1% EtOH) and ending with buffer B (10 mM TEA + 95% EtOH). Before subjecting to bioassays, the solute of the fractions and the buffer components were evaporated by freeze drying. Hydrophobic interaction yielded a good resolution. SIF was eluted with 30-50% EtOH. All activity was concentrated in a few fractions and was separated from fractions containing toxic components.

Collection and purification of SIF for treatment of multi-headed polyps

SIF produced and released from altogether 100 000 primary polyps was collected by solid phase extraction and purified by hydrophobic interaction chromatography. Each second fraction was used to determine the position and width of the activity peak. The residual pooled active fractions were concentrated by lyophilization. The product was dissolved in 0.5 ml methanol and poured into a 5ml polystyrol dish; the methanol was evaporated with a stream of N_2 and a batch of free floating multi-headed polyps added (see Results).

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