Signalling by the FGFR-like tyrosine kinase, Kringelchen, is essential for bud detachment in *Hydra vulgaris*

Stefanie Sudhop[1], Francois Coulier[2], Annette Bieller[1], Angelika Vogt[1], Tobias Hotz[1] and Monika Hassel[1,*]

[1]Philipps University Marburg, FB 17, Morphology and Evolution of Invertebrates, Karl von Frisch Strasse 8, 35032 Marburg, Germany
[2]INSERM Unité 119, 27 boulevard Lei Roure, 13009 Marseille, France
*Author for correspondence (e-mail: hassel@staff.uni-marburg.de)

Accepted 6 May 2004

Development 131, 4001-4011
Published by The Company of Biologists 2004
doi:10.1242/dev.01267

Summary

Signalling through fibroblast growth factors (FGFR) is essential for proper morphogenesis in higher evolved triploblastic organisms. By screening for genes induced during morphogenesis in the diploblastic *Hydra*, we identified a receptor tyrosine kinase (*kringelchen*) with high similarity to FGFR tyrosine kinases. The gene is dynamically upregulated during budding, the asexual propagation of *Hydra*. Activation occurs in body regions, in which the intrinsic positional value changes. During tissue displacement in the early bud, *kringelchen* RNA is transiently present ubiquitously. A few hours later – coincident with the acquisition of organiser properties by the bud tip – a few cells in the apical tip express the gene strongly. About 20 hours after the onset of evagination, expression is switched on in a ring of cells surrounding the bud base, and shortly thereafter vanishes from the apical expression zone. The basal ring persists in the parent during tissue contraction and foot formation in the young polyp, until several hours after bud detachment. Inhibition of bud detachment by head regeneration results in severe distortion, disruption or even complete loss of the well-defined ring-like expression zone. Inhibition of FGFR signalling by SU5402 or, alternatively, inhibition of translation by phosphorothioate antisense oligonucleotides inhibited detachment of buds, indicating that, despite the dynamic expression pattern, the crucial phase for FGFR signalling in *Hydra* morphogenesis lies in bud detachment. Although Kringelchen groups with the FGFR family, it is not known whether this protein is able to bind FGFs, which have not been isolated from *Hydra* so far.

Key words: FGFR, Cnidaria, Budding, Morphogenesis, *Hydra*

Introduction

The superfamily of fibroblast growth factor receptors (FGFRs) comprises structurally similar receptor tyrosine kinases (RTKs) that are activated after interaction with FGFs and heparan sulphates (Ornitz, 2000). As is true for other RTKs, ligand-induced dimerization is the key event for the activation of FGFRs, in which transautophosphorylation on conserved tyrosine residues in the cytoplasmic domain occurs (Mohammadi et al., 1997). The phosphotyrosine residues either stimulate the intrinsic catalytic activity of the receptor or they serve as recruitment sites for downstream signalling proteins such as PLCγ. Phosphorylation of docking proteins recruits additional signalling proteins and induces, for example, activation of the Ras/MAPK pathway (Kouhara et al., 1997). FGFR exert pleiotropic effects on cultured cells and embryonic tissues of mainly mesodermal origin in vertebrates, *C. elegans* and *Drosophila* (Skaer, 1997; Affolter and Shilo, 2000; Borland et al., 2001). Particularly important for FGFR function are three Ig-like loops in the extracellular ligand binding domain, of which loops II and III form the binding sites for FGF ligands and mediate ligand-induced dimerization. Mutations, particularly in loop III, of the four classical vertebrate FGFR are responsible for congenital malformations of bone, limb, kidney and lung, as well as shifted boundaries in the CNS (Burke et al., 1998; Yamaguchi and Rossant, 1995); the function of a fifth and sixth vertebrate FGFR is under investigation (Sleeman et al., 2001; Trueb et al., 2003). Knockout mutants of the two *Drosophila* FGFRs result in failure of heart (mesodermal) or tracheal and glial (ectodermal) development (Skaer, 1997); the *C. elegans* FGFR Egl15 controls migration of sex myoblasts (Borland et al., 2001). Two recently described platyhelminth FGFRs (Ogawa et al., 2002) control stem cell properties; an aberrant FGFR without kinase domain restricts brain tissue to the head region (Cebria et al., 2002). Recurrent, and potentially ancient, functions in triploblastic organisms are thus in the control of cell migration, branching morphogenesis and boundary formation. This raises the question of whether FGFR already exists in the simple organized *Hydra* where specialized cells form only two epithelia but no organs (yet).

Here, we present data on a cnidarian FGFR-like RTK, Kringelchen, which has been isolated from the freshwater polyp *Hydra* and shares typical features with the FGFR of higher metazoa. Functional data indicate that Kringelchen is essential for boundary formation and tissue constriction, which are prerequisite for proper bud detachment.

Materials and methods

Animal culture and expression screening

All methods for the culture of animals, regeneration assays, in situ hybridization of single cell preparations and whole mounts were as described earlier (Hassel et al., 1998; Grens et al., 1996), but for single cell preparations, the heating step to destroy endogenous alkaline phosphatase activity was prolonged to 10 minutes at 85°C. The *Hydra* medium was prepared in MilliQ water. A directionally cloned λ ZAP (Stratagene) cDNA library was established using poly(A^+)-RNA prepared with the Quickprep Micro Kit (Pharmacia) from polyps treated for 4 days in 1 mM LiCl (Hassel et al., 1993). *Kringelchen* was identified by a gene expression screening (Gawantka et al., 1998), in which we picked 25 cDNAs at random from this cDNA library. Their inserts were isolated by PCR using T3 and T7 primers, and transcribed into digoxigenin-labelled antisense RNA (Roche). The expression pattern of the individual cDNAs was analyzed by whole-mount in situ hybridization. Hybridization of the Southern blot was performed at 42°C in 50% formamide with stringent washes in 2× and 0.1× SSC at 42°C.

Sequence analysis and database searches

The DNA sequence was determined by primer walking using an ^{35}S-based sequencing kit (Amersham). Translation of the nucleotide sequence into amino acids, mapping, determination of the pKa and homology searches were performed with the HUSAR program package (German Cancer Research Institute, Heidelberg). For phylogenetic analysis, a recently established database was used, which is available under http://pbil.univ-lyon1.fr/RTKdb (Grassot et al., 2003).

Treatment with SU5402

Sixty animals per experiment were incubated in a final concentration of 10 μM SU5402 (Calbiochem), 1 mM ATP and 0.1% DMSO in *Hydra* medium for 24 hours. Control incubations were performed in the same solution without SU5402. The stock solution of SU5402 (10 mM in DMSO) was stored in aliquots at –20°C. Fresh ATP solution was prepared for each experiment.

Antisense experiments

Mixed phosphorothioate antisense oligonucleotides were synthesized and HPLC-purified by Eurogentec. They were designed for optimal function according to Brysch and Schlingensiepen (Brysch and Schlingensiepen, 1994): Phosphorothioate oligonucleotides complementary to the 5′ coding region of the *kringelchen* cDNA were: oligo 1 (165-182 corresponding to nucleotides 65-82 of the coding region, thioated nucleotides are marked with an asterisk) AAT T*A*A* CTG GCT CT*G T*A*A; and oligo 2 (217-234 corresponding to position 117-134 of the coding region) TGA A*G*C* T*GG CAG TA*G A*T*C*. In the semi-random mismatch controls nucleotides in 3 or 4 positions were exchanged for each other. This prohibits specific interaction without changing the base composition and is preferable to sense controls. In mismatch control Oligo 1c, for example, nucleotide 3 was changed to position 6, 6 to 10, 10 to 14 and 14 to 3 (the exchanged nucleotides are underlined): AA<u>C</u> T*A*<u>T</u>* CGT <u>A</u>CT C<u>G</u>*G T*A*A*. In mismatch control Oligo 2c, nucleotide position 4 was changed to 7, 7 to 12 and 12 to 4: TGA <u>G</u>*G*C* <u>A</u>*GG CA<u>A</u> TA*G A*T*C*. The rationale for the complex design of the four antisense oligonucleotides was as follows. Three unmodified nucleotides at the 5′ end allowed phosphorylation to control the quality of the oligonucleotides in a 20% sequencing gel. The next three or four nucleotides (4-7) were phosphorothioated to protect against nuclease activity, the next 6 or 7 nucleotides (7-13) were left unmodified to allow for RNase H cleavage of the resulting hybrid, nucleotide 15 was left unmodified to avoid a long stretch of phosphorothioates, which is known to increase unspecific stickiness, and the last 3 nucleotides (16-18) at the 3′ end were thioated again to protect from nuclease digestion. The antisense oligonucleotides were electroporated in 20 whole animals (100 μl volume, 4 mm cuvettes) using an Easyject Plus Electroporator (Equibio) at the following settings: 1500 μF, 400 V, 99 Ω. After electroporation, polyps were stored at 4°C overnight and thereafter kept at 18°C. From 5×20 electroporated polyps (three independent experiments) the 39 survivors were pooled for Fig. 4H. The electroporation protocol was adapted from Lohmann et al. (Lohmann et al., 1999) and yielded consistent results, while incubations with DOTAP (Boehringer) were less reliable.

Tissue sections

For tissue sections, polyps were embedded after in situ hybridization in 1% agar-agar. Agar blocks with single polyps were excised and equilibrated in 70% ethanol overnight, followed by two washes in dioxane for 45 minutes. After the second wash, half of the dioxane was removed and replaced by Spurr Standard plastic embedding medium: 13 g NSA (nonenyl succinic anhydride, Agar Scientific), 5 g ERL (4-vinylcyclohexene dioxide, Polysciences), 4 g DER (diglyoidyl ether of polypropylene glycol, Agar Scientific), 0.2 g S-1 (dimethylaminoethanol, Agar Scientific). The agar blocks were equilibrated in this mixture for 90 minutes, then again half of the solution was removed and replaced by Spurr Standard (90 minutes incubation time). An overnight incubation in pure Spurr Standard followed. The next day, the embedding medium was renewed. After 6-8 hours, the agar blocks were transferred in forms filled with the plastic embedding medium. Polymerization occurred at 70°C for 16 hours. Tissue sections (2 μm) were cut with a glass knife on a Pyramitome (LKB Biotech) and transferred to a glass slide in a drop of water.

Results

Isolation of a putative FGFR encoding gene and sequence features

The *Hydra* FGFR-like gene *kringelchen* was isolated from a lithium-induced *Hydra vulgaris* cDNA library, in which genes involved in foot formation are likely to be overexpressed (Hassel et al., 1993). The name *kringelchen* was deduced from the expression pattern in lithium-treated and control animals with recently detached buds, where small rings of *kringelchen*-expressing cells were detected (see Fig. 3I,M) (*kringelchen* from the German nickname for small rings). The full length clone (2847bp, GenBank Acc. No. AY193769) carries the recently identified splice leader B (Stover and Steele, 2001). The ORF of 2448 base pairs encodes a protein of 816 amino acids with a deduced molecular weight of 93,4 kDa (Fig. 1A). The Kringelchen protein contains a signal sequence (VVLVLLMSRLVFG) at position 7-19, three putative Ig-like loops (D1, position 44-95; D2, position 147-201; D3, position 250-324), an intervening (D1-D2) stretch of acidic amino acids, a single putative transmembrane domain (position 369-393) and a highly conserved bipartite catalytic domain of a receptor tyrosine kinase (position 472-757).

BLAST search places the protein within the family of fibroblast growth factor receptors (FGFR), for which a bipartite intracellular tyrosine kinase domain is typical (Fig. 1A,D). The ATP binding domain consensus GEGAFGRV-----TVAVK lies between amino acids 481 to 508. Several functionally important intracellular autophosphorylation sites are conserved in Kringelchen: the pair Y642/Y643 corresponds to Y653/Y654 in vertebrate FGFR1 and is essential for activation of the kinase domain. Y755 in Kringelchen corresponds to

Y766 in FGFR1, which is the main phosphorylation site for the interaction with PLCγ (Mohammadi et al., 1991). Of relevance for downstream signalling are also consensus sequences for potential interaction sites for SH2-binding domains in the juxtamembrane position 408 to 411 (**Y**IK**P**, interaction with Src) and 719 to 722 (**Y**TL**M**, see Fig. 1D), a consensus for SH3-binding domains is in positions 760 to 763 (**P**IQ**P**).

The features of the extracellular, ligand-binding domain of this ancient FGFR-like protein are particularly interesting: Usually, three Ig-like loops are present: the function of D1 is still unclear, D2 and D3 bind FGF, and D3 conveys FGF binding specificity. In *Hydra*, Ig-like loops D1 and D2 are likely to be formed by four highly conserved cysteines (positions 43, 94, 147 and 201; Fig. 1B,C). As in higher evolved FGFR, the intervening region is acidic (pKa=4.14), but the acidic residues are not clustered. Most conspicuous, however, is the region corresponding to D3: although usually cysteines clamp Ig-like loops, the alignment indicates that in the case of the putative Kringelchen D3 two hydrophobic amino acids might take this role, i.e. Phe324 and Tyr250.

Southern analysis (Fig. 2A) shows that *kringelchen* is encoded by a single copy gene. On a northern blot (not shown), a single mRNA about 3.8 kb in size was detected. The discrepancy between mRNA size and the obviously full-length cDNA (Fig. 1A) indicates that internal poly(T) priming might have occurred in the 3′ UTR.

Phylogenetic analysis confirms that Kringelchen is an ancient FGFR

Phylogenetic analysis (distance method) was carried out using the kinase domain of 90 receptor tyrosine kinases and comprised representatives of each RTK class (Grassot et al., 2003) from nine species representative of the major bilateria taxa. This analysis groups Kringelchen within FGFR (class IV), VEGFR (class V) and PDGFR (class III) families (not shown). A close-up phylogenetic analysis using sequences from class III, IV and V (Fig. 2B) places Kringelchen at the base of the FGFR family (bootstrap value of 72). Analysis using a parsimony algorithm gave similar topologies.

Taken together, Kringelchen is a very good candidate for an archetype FGFR with highly conserved, but also clearly distinct, features as compared with higher evolved FGFR in triploblasts.

Kringelchen is dynamically expressed in all phases of bud evagination, differentiation and detachment

Well-fed *Hydra* propagate mainly asexually by budding. Bud formation starts with the determination of a bud field in the mid gastric region, followed by recruitment of tissue from the gastric column, which is displaced into the evaginating early bud tissue. About 24 hours after the first signs of bud evagination, head structures start to form in the apical part. Shortly thereafter, the bud base begins to contract, foot tissue is formed and, finally, about 4 days after evagination, a fully differentiated young polyp detaches from the parent [for a detailed morphological analysis of the budding process and staging see Otto and Campbell (Otto and Campbell, 1977)]. As the budding process activates the main developmental programs of a *Hydra*, expression screening using whole-mount in situ hybridization on budding polyps is an easy-to-use tool to isolate genes involved in morphogenesis. *Kringelchen* was identified as a potentially interesting gene by its astonishingly dynamic RNA expression pattern, which distinguishes five phases (Fig. 3).

Early evagination phase

Until stage 3 (up to 3 hours after evagination) *kringelchen* RNA is detectable only at the evagination site, from which the bud develops. At stage 2, *kringelchen* RNA is localized in a spot of about 60 epithelial cells (see Fig. 3Q), then spreads proximally and is, at stage 3, expressed in the ecto- and endoderm of the growing protrusion (Fig. 3A,B). Expression of *kringelchen* is always restricted to epithelial cells (see Fig. 3R,S).

Middle evagination phase

When the bud starts to elongate (stage 4), a spot-like zone of strong expression forms in the tip endoderm. Only a low level of RNA is detected in the growing body column (Fig. 3C-E). This changes as soon as the length of the bud approaches its vertical extension: from now on, *kringelchen* RNA is additionally found to be upregulated in a ring of ectodermal cells surrounding the bud base (Fig. 3E-L,P).

Late evagination phase

In stage 5, when the bud length exceeds its vertical extension, the signal in the ring-like zone intensifies and co-exists for a short time, together with the apical patch of expressing cells (Fig. 3G,H). By close-up microscopy of three bud bases, the mean number of ectodermal cells per ring was determined to be 171.4±33.2.

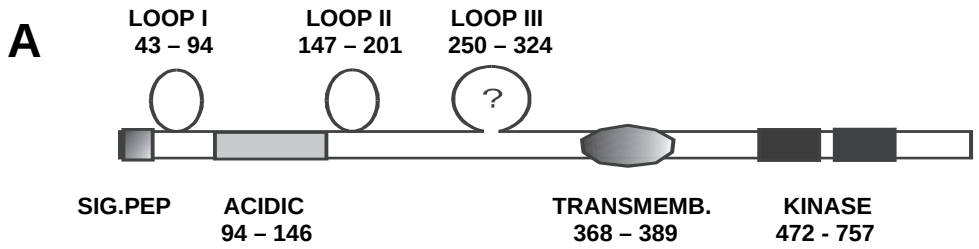
Differentiation phase

As soon as the earliest signs of tentacle buds are visible (stage 6, about 20 hours after evagination), the *kringelchen* signal is completely lost from the apical tip (Fig. 3I,K). The circular zone of expression surrounding the now narrowing bud base, remains intensive for the next 3 days (Fig. 3I-L) and is about five or six cells thick. Both parent and bud tissue express the gene in the ectoderm until shortly before detachment (Fig. 3K), when the ring has narrowed to two or three cells (Fig. 3L). Sections (2 μm) showed that shortly before detachment endoderm, mesogloea and ectoderm of the parent on the one side, as well as endoderm and mesogloea of the bud on the other side form closed sheets. The bud ectoderm, by contrast, is not yet closed at the prospective foot end and columnar cells sit ‘waiting’, well arranged and right above the detachment zone (see arrowhead in Fig. 3L).

Detachment phase

When the bud is ready to detach (as seen by the narrow tissue bridge connecting it to the parent), *kringelchen* RNA is no longer detectable in bud tissue by in situ hybridization, but persists in the parent (Fig. 3L). After detachment (Fig. 3M), about 40 *kringelchen*-expressing cells remain as a small ring in the ectoderm of the parent polyp for a short time. The ring-like expression zone constricts quickly to a patch of cells (Fig. 3N-O) and within 1-2 hours expression is switched off – first in single cells, then in all of them.

As new buds always form above old ones, polyps with one new and one recently detached bud show the early diffuse

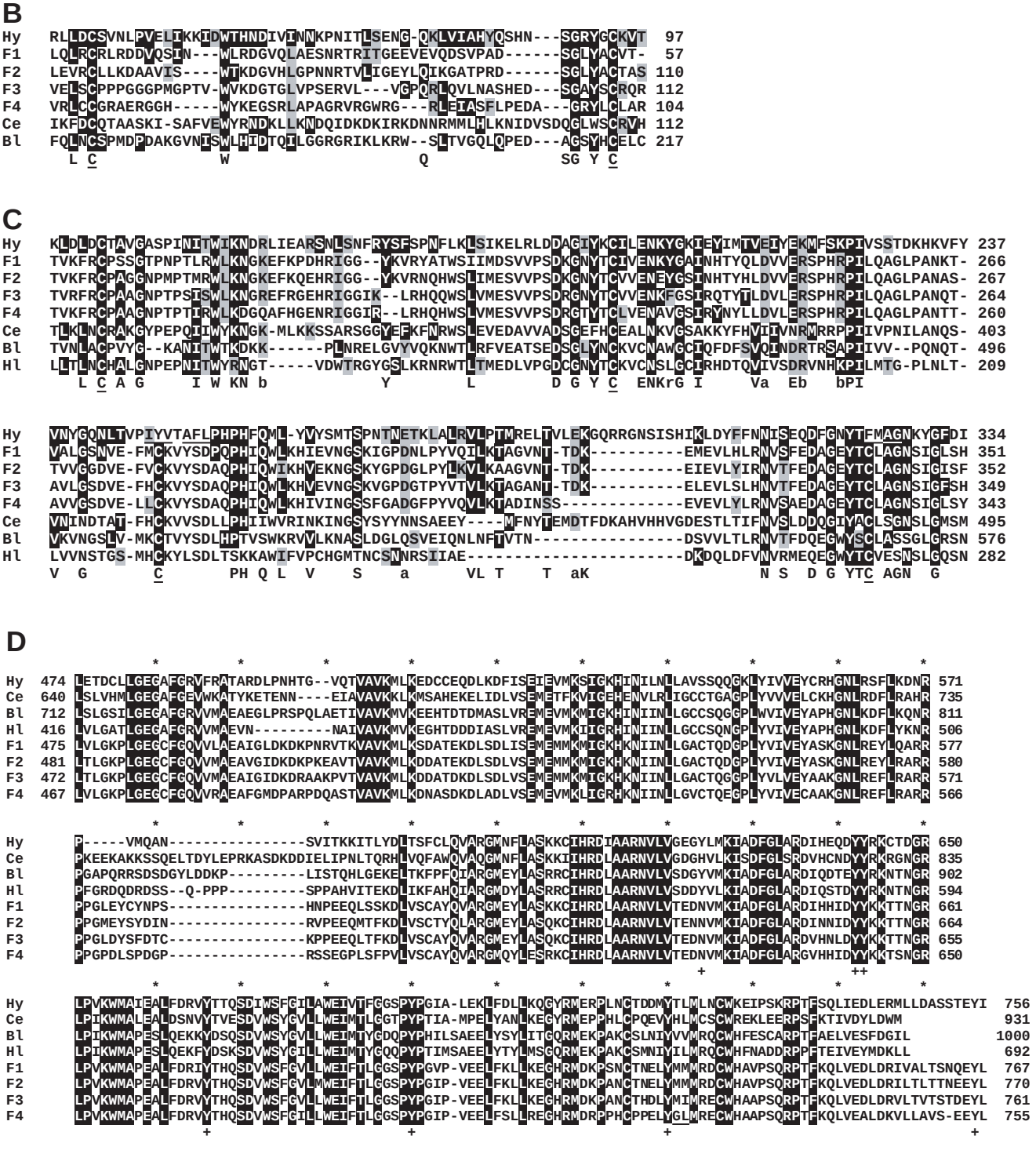



```
TTTTAGTCCCTGTGTAATAAGATTTTATTGTTTATAAAAAAAAAAAAATTGTTGGATTTAATGCTGTGTATTTATAAATACAAATGTTGTA   90
TATAGCAAAATGATATCAGATTGGTGTGTTGTTTTGGTTTTATTAATGTCAAGATTAGTTTTTGGGCTGAATTTTACAGAGCCAGTTAAT  180
          M  I  S  D  W  C  V  V  L  V  L  L  M  S  R  L  V  F  G  L  N  F  T  E  P  V  N   27
TATATATTGAAATTAGGAGAAGATAGTTCATCTAGACTATTAGATTGTTCAGTTAATCTTCCTGTTGAATTAATCAAAAAAATTGATTGG  270
Y  I  L  K  L  G  E  D  S  S  S  R  L  L  D  C  S  V  N  L  P  V  E  L  I  K  K  I  D  W   57
ACACACAATGATATTGTTATCAATAATAAACCTAATATAACATTATCTGAAAATGGTCAAAAACTTGTTATTGCTCATTACCAATCCCAT  360
T  H  N  D  I  V  I  N  N  K  P  N  I  T  L  S  E  N  G  Q  K  L  V  I  A  H  Y  Q  S  H   87
AATTCTGGTCGTTATGGGTGCAAAGTAACAGCTATGAATGAAGAATCTGTTCAAAGGGTATTTGATCTACTGCCAGCTTCAGAAACTGAG  450
N  S  G  R  Y  G  C  K  V  T  A  M  N  E  E  S  V  Q  R  V  F  D  L  L  P  A  S  E  T  E   117
GGAAACCAAACCATTGAAATGATGCTGAGAATAAAAAATGACATATCGTTACTTGTTGAGTTAGTGATGAATAAATTAGATTTAGATTGT  540
G  N  Q  T  I  E  M  M  L  R  I  K  N  D  I  S  L  L  V  E  L  V  M  N  K  L  D  L  D  C   147
ACCGCTGTAGGTGCTTCACCTATTAATATTACATGGATAAAAAATGATCGGCTGATAGAAGCAAGGTCCAACTTGTCTAATTTTCGTTAT  630
T  A  V  G  A  S  P  I  N  I  T  W  I  K  N  D  R  L  I  E  A  R  S  N  L  S  N  F  R  Y   177
TCTTTCTCACCAAATTTTTTGAAATTAAGTATAAAAGAATTGCGACTTGATGATGCTGGTATTTACAAATGTATTTTGGAAAATAAATAT  720
S  F  S  P  N  F  L  K  L  S  I  K  E  L  R  L  D  D  A  G  I  Y  K  C  I  L  E  N  K  Y   207
GGCAAGATAGAACATATAATGACTGTTGAAATATATGAGAAAATGTTCTCTAAACCAATAGTATCTTCTACAGACAAACATAAAGTTTTT  810
G  K  I  E  H  I  M  T  V  E  I  Y  E  K  M  F  S  K  P  I  V  S  S  T  D  K  H  K  V  F   237
TATGTCAACTATGGTCAGAATTTAACTGTCCCTATATATGTTACTGCATTTCTACCCCACCCTCACTTTCAAATGTTATACGTTTACAGC  900
Y  V  N  Y  G  Q  N  L  T  V  P  I  Y  V  T  A  F  L  P  H  P  H  F  Q  M  L  Y  V  Y  S   267
ATGACAAGTCCAAATACAAATGAAACTAAATTGGCACTTAGGGTATTACCAACAATGAGAGAACTAACTGTCTTGGAGAAAGGCCAGAGA  990
M  T  S  P  N  T  N  E  T  K  L  A  L  R  V  L  P  T  M  R  E  L  T  V  L  E  K  G  Q  R   297
AGAGGCAATTCAATTTCTCATATCAAGCTAGATTATTTTTTCAATAATATATCAGAGCAAGATTTTGGTAATTATACCTTTATGGCTGGA 1080
R  G  N  S  I  S  H  I  K  L  D  Y  F  F  N  N  I  S  E  Q  D  F  G  N  Y  T  F  M  A  G   327
AATAAGTATGGTTTTGATATATACCCGTTTCAAATATTGCATACAAAATATATGCAAACAACTGTCTTCCCGCCAATGAAATCAAGTATT 1170
N  K  Y  G  F  D  I  Y  P  F  Q  I  L  H  T  K  Y  M  Q  T  T  V  F  P  P  M  K  S  S  I   357
AATAAAATCTACAAAGAGGAATCCGTAGAAAAAACAGTTATTTTTATTGTCATAACTTCAATGCTTGCTGGGCTAATTTTTGTTGCATTT 1260
N  K  I  Y  K  E  E  S  V  E  K  T  V  I  F  I  V  I  T  S  M  L  A  G  L  I  F  V  A  F   387
GTTATTTTTTTATTTGTCGTGTTCGTAGCAAAGACAAATTTAAAAACAGTAACATCAACTACATCAAACCTTTAGAAACTGTGATTCTT 1350
V  I  F  F  I  C  R  V  R  S  K  D  K  F  K  N  S  N  I  N  Y  I  K  P  L  E  T  V  I  L   417
AATCTTGGGGATAATAACACTAGTGGTGTTACCATGGTTACTTCTGTCTCTGCTTCCTACTGCAGTCGGCGTTTCAGACATTCACTTAAT 1440
N  L  G  D  N  N  T  S  G  V  T  M  V  T  S  V  S  A  S  Y  C  S  R  R  F  R  H  S  L  N   447
AATAATTTAATAAATGATAAGCAAAAATTGAATTTAAAAATAGCTCCAGATCCTGCTTGGGAGATAAAACTTGAACAATTAGAAACAGAT 1530
N  N  L  I  N  D  K  Q  K  L  N  L  K  I  A  P  D  P  A  W  E  I  K  L  E  Q  L  E  T  D   477
TGTTTATTGGGAGAGGGAGCTTTTGGTAGAGTTTTTCGTGCAACAGCAAGAGATTTACCAAATCACACTGGAGTTCAAACAGTTGCTGTC 1620
C  L  L  G  E  G  A  F  G  R  V  F  R  A  T  A  R  D  L  P  N  H  T  G  V  Q  T  V  A  V   507
AAAATGCTGAAAGAAGATTGTTGTGAACAAGATTTAAAAGATTTTATATCTGAAATAGAAGTAATGAAATCTATAGGAAAACATATCAAT 1710
K  M  L  K  E  D  C  C  E  Q  D  L  K  D  F  I  S  E  I  E  V  M  K  S  I  G  K  H  I  N   537
ATTCTGAATTTACTTGCAGTGTCATCTCAGCAAGGAAAGTTATATATAGTGGTTGAATATTGTCGTCATGGTAATTTGCGCTCTTTTCTG 1800
I  L  N  L  L  A  V  S  S  Q  Q  G  K  L  Y  I  V  V  E  Y  C  R  H  G  N  L  R  S  F  L   567
AAAGACAATCGACCTGTTATGCAAGCTAATTCTGTGATAACAAAAAAAATAACATTGTACGATTTAACGTCGTTTTGCTTACAAGTTGCA 1890
K  D  N  R  P  V  M  Q  A  N  S  V  I  T  K  K  I  T  L  Y  D  L  T  S  F  C  L  Q  V  A   597
AGAGGAATGAATTTTTTAGCTTCAAAAAAGTGCATCCATCGAGACATAGCTGCTCGAAATGTTCTTGTTGGTGAAGGTTATTTAATGAAA 1980
R  G  M  N  F  L  A  S  K  K  C  I  H  R  D  I  A  A  R  N  V  L  V  G  E  G  Y  L  M  K   627
ATTGCTGATTTTGGACTTGCACGTGATATTCATGAACAAGATTATTATAGAAAATGCACAGATGGGCGATTACCCGTCAAATGGATGGCA 2070
I  A  D  F  G  L  A  R  D  I  H  E  Q  D  Y  Y  R  K  C  T  D  G  R  L  P  V  K  W  M  A   657
ATTGAAGCTTTGTTCGATCGTGTTTATACTACTCAAAGTGATATATGGTCATTTGGAATTTTGGCATGGGAAATTGTAACATTTGGTGGA 2160
I  E  A  L  F  D  R  V  Y  T  T  Q  S  D  I  W  S  F  G  I  L  A  W  E  I  V  T  F  G  G   687
TCGCCTTACCCTGGAATTGCATTAGAAAAATTGTTTGATTTATTAAAGCAAGGCTATCGAATGGAAAGACCACTTAATTGTACTGATGAT 2250
S  P  Y  P  G  I  A  L  E  K  L  F  D  L  L  K  Q  G  Y  R  M  E  R  P  L  N  C  T  D  D   717
ATGTATACACTTATGCTGAATTGTTGGAAAGAAATCCCATCAAAACGTCCGACATTTTCACAACTAATTGAAGATCTAGAAAGAATGCTA 2340
M  Y  T  L  M  L  N  C  W  K  E  I  P  S  K  R  P  T  F  S  Q  L  I  E  D  L  E  R  M  L   747
TTGGATGCAAGTTCTACAGAATATATAGATCTTCAACCAATTCAACCAGAACGCACAGAGTCATTTTCAACATCATTACACACTTCTGCA 2430
L  D  A  S  S  T  E  Y  I  D  L  Q  P  I  Q  P  E  R  T  E  S  F  S  T  S  L  H  T  S  A   777
AGCATGTTGAATACAGATTTGCACGAGAAAAATAAATGTGATCATGACGAAATATCCTTCACTCATGAAGATGGTTTATCAGAAGCAGAT 2520
S  M  L  N  T  D  L  H  E  K  N  K  C  D  H  D  E  I  S  F  T  H  E  D  G  L  S  E  A  D   807
ATTCTTCTTAGTCACTATGCAGTTTCTTAATTACTTCAACTGCTTGTGACGTTTTTAGATGTAAACTTGATTTATCAGTTTCTCTAAATG 2610
I  L  L  S  H  Y  A  V  S  *                                                               816
ATTGCCTTGTCATTAACGTGTATTGCATTTGACTTTCATCATGAAGAATCTAGAGATCTATCCGATTCTTCTCTATTAATAAACACTGTC 2700
ATGAAGATTGTTTATATATGAATCATTGGATCAGGAACTTCTTGAGGATCATAATAAAAGTAATACTAAATATGAAATTGCCAGCTAAAA 2790
CCAAAGGTTCGTAATTTTCGATGCGAATAAATGAGCTTTTCAAAAAAAAAAAAAAA                                   2846
```

Fig. 1. Sequence features of Kringelchen (GenBank Accession Number, AY193769). (A) Proposed structure and full-length sequence: features and their corresponding amino acid positions are given. The partial sequence of splice leader B is underlined. The amino acid sequence contains a signal peptide (boxed and italic), a transmembrane domain (boxed and bold) and an intracellular kinase domain (boxed only). Ig-like loop I and Ig-like loop II are boxed and shaded, and the sequence between Tyr 250 and Phe324 (corresponding to Ig-like loop III) is shaded only. The highly conserved cysteines, which form the Ig-like loops I and II are boxed and shaded. SH2 and SH3 domain-binding consensuses are bold and underlined, bold tyrosines correspond to crucial autophosphorylated tyrosines in vertebrate FGFR. (B,C) Alignment of the *Hydra* sequence corresponding to the first, second and third putative Ig-like loops with vertebrate and invertebrate FGFR. (B) Ig-like loop I (D1), (C) Ig-like loop II (D2; upper part of the figure) and Ig-like loop III (D3; lower part of the figure). a, acidic amino acid; b, basic amino acid; r, aromatic amino acid. (D) Intracellular (kinase) domain. Underlined is a potential SH2-binding site; (auto)phosphorylation sites (identified in vertebrate FGFR) are marked with +. Shortcuts and Accession Number for the used sequences are: Hy, *Hydra vulgaris* Kringelchen (AY193769); Ce, *C. elegans* Egl 15 (AAC46934); Bl and Hl, *Drosophila* Breathless (Q09147) and Heartless (Q07407); F1-F4, human FGFR 1-4 (P11362, P21802, P22607, P22455).

expression in evaginating buds plus a ring or a patch of expression in a more basal position (Fig. 3I).

Detachment of the young polyp seems to leave a crater in the ectoderm of the parent (Fig. 3M): the ring of *kringelchen*-positive cells is elevated above the level of tissue within this ring. This raised the possibility that the detaching bud partially removes the ectoderm of the parent leaving the naked mesogloea behind. Detailed analysis of the detachment site in 2 μm sections showed, however, that the detachment site is covered by flattened ectodermal cells, which might in the



following contract and close the 'gap' to bring the *kringelchen*-positive cells back in the normal tissue environment.

The dynamic expression pattern could be caused by overlapping detection of two very closely related kinases. Control experiments with separate antisense RNA probes for the extra- and the intracellular parts of *kringelchen* yielded identical results, which excludes the possibility of crossreactivity with a related sequence.

In situ hybridization to single cell preparations (Fig. 3R,S) detects *kringelchen* expression exclusively in ecto- and/or endodermal epithelial cells (localization depending on the bud stage, see above). Overexposure detects *kringelchen* in up to 40% of the epithelial cells, which explains why RT-PCR detects transcription of the full-length sequence of *kringelchen* at a low level all over the body (not shown).

Correct *kringelchen* expression is tightly coupled to proper bud detachment

The dynamic expression pattern in morphogenetically active

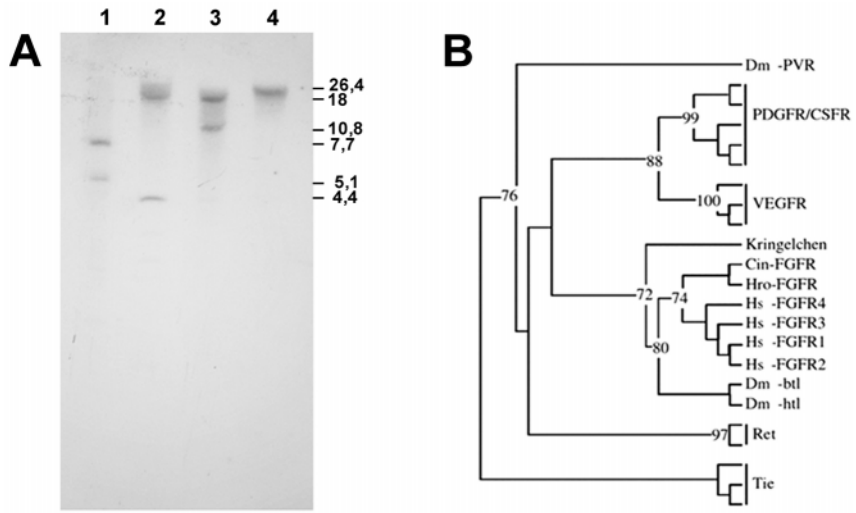


Fig. 2. Southern blot and phylogenetic tree. (A) Southern blot probed with the full-length kringelchen cDNA. Genomic DNA (10 μg) was digested to completion with *Hin*dIII (lane 1), *Nco*I (lane 2), *Eco*RV (lane 3) and *Xho*I (lane 4). *Hin*dIII, *Nco*I and *Eco*RV cut once within the coding sequence (positions 2076, 1383 and 101 of the full-length cDNA, respectively); *Xho*I does not cut. The size was determined using a λ/*Hin*dIII digested DNA size marker. (B) Phylogeny of class III, IV, V and XII receptor tyrosine kinases (Grassot et al., 2003). Kinase domain sequences from RTK Class III (CSFR/PDGFR), IV (FGFR), V (VEGFR) and XII (Ret) were used for phylogeny inference (distance method). Class IX (Tie) sequences were used as outgroup. Node with bootstrap values over 50 are indicated (500 bootstrap replicates). Dm, *Drosophila melanogaster*; Hs, *Homo sapiens*; Hro, *Halocynthia roretzi*; Cin, *Ciona intestinalis*; btl, *Drosophila* breathless FGFR; htl, *Drosophila* heartless FGFR. Kringelchen branches at the base of the FGFR tyrosine kinase superfamily.

regions during all phases of budding suggested that *kringelchen* participates in general bud development. In order to analyse if interference with bud detachment alters the expression pattern, we performed a competition experiment: early budding and head regeneration compete for resources, which is indicated by the fact that the growing bud prohibits head regeneration and takes over the axis without detaching (Tardent, 1972). Therefore, we investigated the *kringelchen* RNA expression pattern in regenerating, budding polyps. Either head or foot regeneration of the parent was induced by dissection of the polyp right above or below the developing bud (Fig. 4A,H). Polyps of our *Hydra vulgaris* strain bearing buds up to stage 4 are unable to regenerate a head if the head and upper body region of the parent are removed immediately above an early (stage 3) bud (Fig. 4A). The bud takes over the axis of the parent in up to 100% of the cases. For a detailed analysis of the *kringelchen* expression pattern in such secondary axes, budding polyps were dissected early (stage 3) and late (stage 8). Figure 4B shows that the early RNA expression in the bud tip remains unaffected by regeneration of the parent *Hydra*. But once the bud reaches stage 5-6, when usually the ring of *kringelchen*-expressing cells forms at the bud base, this circular expression zone was found to be severely distorted, broken up into patches or even completely missing (Fig. 4C-G). In addition, many polyps express *kringelchen* RNA at a high level throughout the bud tissue (Fig. 4F), where normally no expression is detectable. This feature seems to be due to the

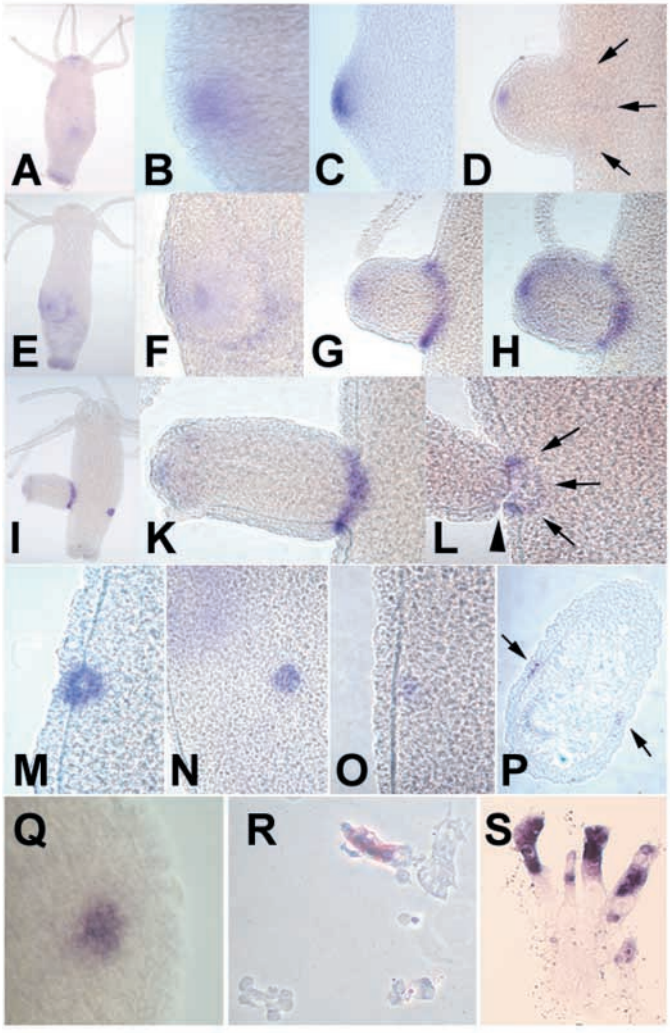


Fig. 3. Expression pattern of *kringelchen* in buds as detected by in situ hybridization with a digoxigenin-labelled antisense probe. Staging of buds is according to Otto and Campbell (Otto and Campbell, 1977). (A,B) Stage 1 (ectoderm thickening and early evagination); (C) stage 3 (about 5 hours after first signs of evagination); (D) stage 4; (E-H) stage 5 (the tentacle in G,H belongs to the parent); (I-K) stage 7 (tentacle buds form); (L) stage 10 (shortly before detachment). (M-O) Budding region of the parent polyp after detachment: (M) immediately after detachment, (N) 30 minutes afterwards, (O) 60 minutes afterwards. (P) Semi-thin section of a stage 5 bud. (Q) Close-up of a stage 2 bud tip, in which about 60 ectodermal cells are distinguishable. (R,S) Single cell preparations of tissue derived from the budding region with *kringelchen*-positive epithelial cells. (R) Phase contrast imaging, allowing the evaluation of non labelled cell types; (S) a group of epithelial cells from the budding region shown by light microscopy.

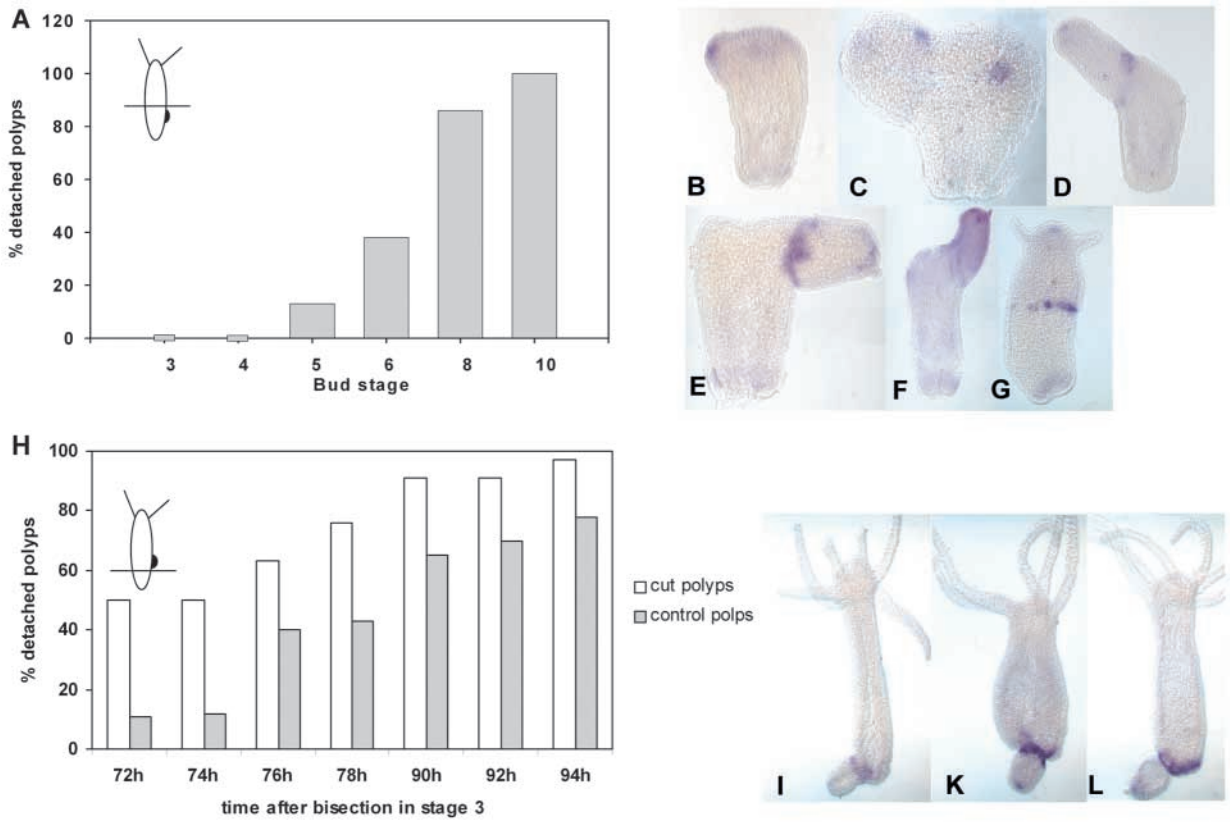


Fig. 4. Effects of head and foot regeneration induced in the parent on bud development and *kringelchen* expression. (A-G) Head regeneration and bud development [bud stages according to Otto and Campbell (Otto and Campbell, 1977)]. (A) Competition of both processes results in failure of bud detachment if regeneration is induced early in budding (*n*=100). (B-G) Expression pattern of *kringelchen* if the parent head is removed immediately above a stage 3 bud: (B) 10 hours, (C) 26 hours, (D) 22 hours, (E) 31 hours, (F) 36 hours, (G) 57 hours. (H-L) Effect of foot regeneration on bud development. (H) Detachment of the young polyp is slightly accelerated (*n*=100 each). (I-L) Expression pattern of *kringelchen* after foot removal immediately below a stage 3 bud, evaluation about 24-25 hours after cutting. The typical ring is present and broader than in normal buds.

regeneration process rather than being relevant for the lack of detachment, because it was also found in buds dissected at stage 8, in which close to 90% of the polyps detach and parent head regeneration occurs normal.

From our experiments, it appeared that proper *kringelchen* expression and bud detachment are tightly linked. Because, in contrast to head regeneration, foot regeneration does not inhibit bud detachment (Tardent, 1972), we removed the foot of budding polyps right below an emerging bud and performed in situ hybridization as a control to validate the specificity of the observed effects. The developing young polyps detached completely normally and even slightly earlier (Fig. 4H). The ring of *kringelchen*-expressing cells remained unaffected and developed in its normal shape (Fig. 4I-L). Interestingly, the hybridization signal appeared more intense and the ring was often thicker than in the normal budding polyps – which perhaps indicates a correlation with the faster detachment. In addition, ubiquitous ectopic expression was never detected in buds developing at foot regenerating parents, which indicates that only apical regeneration of the parent induces 'flooding' of bud tissue with *kringelchen* RNA. This experimental control therefore confirms a tight relation between the integrity of the ring-shaped *kringelchen* expression zone and normal bud detachment.

Inhibition experiments reveal that Kringelchen functions in bud detachment

The dynamic expression pattern of *kringelchen* initially suggested multiple functions during axis formation, patterning, differentiation and detachment of a bud. In order to determine its function, we used two approaches based on (1) the specific biochemical inhibition of FGFR kinase activity, SU5402; and (2) inhibition of *kringelchen* translation by phosphorothioate (PT) antisense oligonucleotides. Both treatments specifically inhibited bud detachment (Fig. 5).

The FGFR-specific inhibitor, SU5402, binds to the FGFR kinase domain. It is highly specific, fails to inhibit other tyrosine kinases like insulin receptor and EGFR and interferes only weakly (above 60 μM) with PDGFR (Mohammadi et al., 1997). In *Hydra*, seven out of the eight amino acids described necessary for SU5402 binding are identical. Treatment with 10 μM SU5402 for 24 hours (Fig. 5A) allowed normal bud development and proportioning, but prevented detachment. The resulting abnormalities are schematically summarized in Fig. 5B. As in the above-described head-bud competition experiments, early bud stages were more susceptible to the inhibitor than were late ones (Fig. 5C): more than 70% of the young polyps treated at stage 3, but only about 60% at stage 5 and 45% at stage 7,

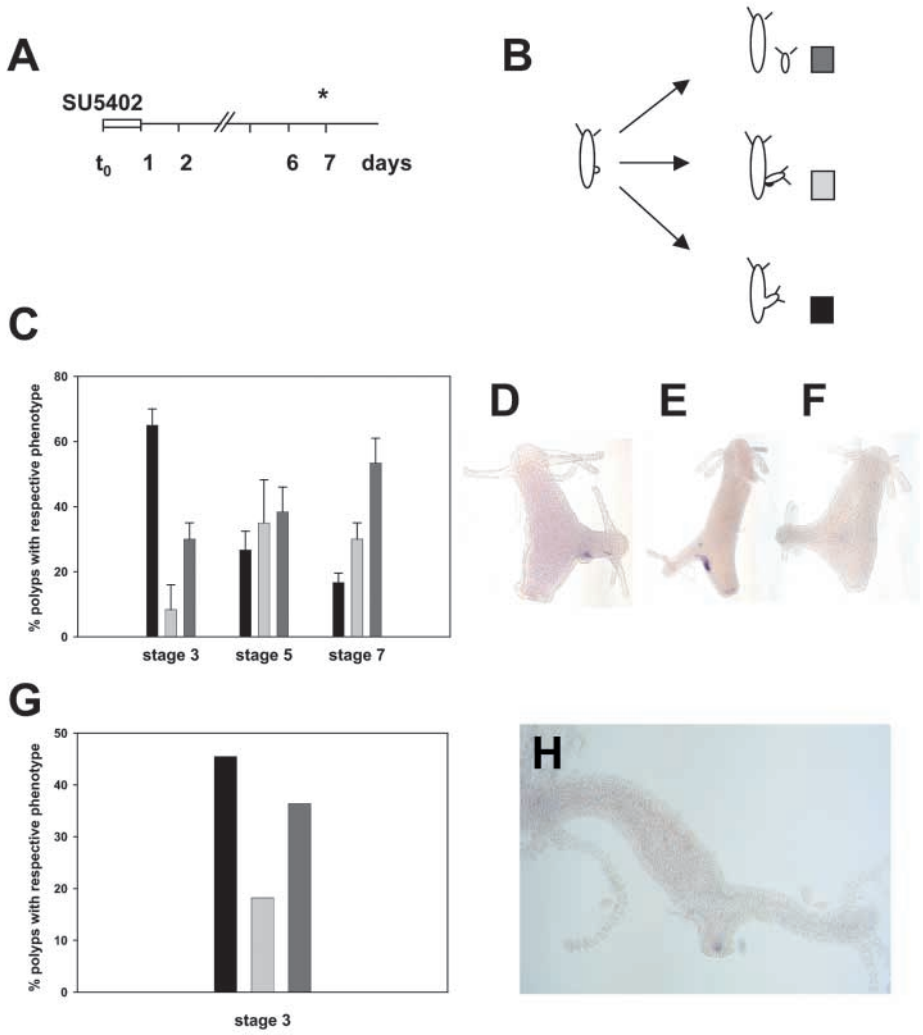


Fig. 5. Inhibition of bud detachment and *kringelchen* expression after treatment with either the FGFR inhibitor SU5402 or phosphorothioated oligonucleotides. (A) Treatment scheme for SU5402. Buds were treated for 24 hours with SU5402 and examined at stages 3, 5 and 7. Asterisk indicates when in situ hybridization was carried out. (B) Scheme of the increasingly severe abnormalities obtained after treatment with SU5402. (C) Effects of SU5402 treatment on the characteristics of the animals at three different stages after treatment. The most severe abnormality predominated at earlier stages after treatment with either SU5402 or phosphorothioate antisense oligonucleotides. (D-F) In situ hybridization of the *kringelchen* antisense RNA probe to *Hydra* with increasingly severe abnormalities produced by SU5402 treatment. (G) Effects of electroporation of PT-antisense oligo 1 in stage 3 buds. Sixty polyps were electroporated, the 39 survivors evaluated. (H) In situ hybridization of *kringelchen* RNA probe to a typical abnormally elongated bud obtained by the antisense oligonucleotide treatment. Evaluation of the abnormalities and in situ hybridization were carried out 7 days after the end of treatment.

failed to detach. SU5402 treatment at stage 3 caused the severest abnormalities, with buds that remained attached to the parent without any sign of foot differentiation (Fig. 5F). Treatment of stages 5 and 7 resulted in a milder abnormality with an increasing amount of buds forming patch feet at the lower side of the bud and showing signs of tissue contraction at the bud base (Fig. 5D,E). Patch feet were identified by their whitish appearance and stickiness, because biochemical detection of the typical peroxidase activity (Hoffmeister and Schaller, 1985) is not possible in parallel with in situ hybridization. The inhibitor had no effect at all if treatment started at stage 8 or later, when the decision for detachment is apparently already fixed.

SU5402 was also used in a treatment of polyps, which were 'pregnant' with buds. Our feeding regime, in which polyps are fed only five times a week, partially synchronizes the culture: after 2 days of starvation, mainly stage 8-10 buds are present, which detach on the second day of feeding resulting in a culture containing mainly big, budless polyps, which are already induced to form new buds by feeding ('pregnant'). Budding is resumed on the third day of feeding by about 80% of the big polyps. Incubation of big, budless polyps (selected from the culture on the second day of feeding) for 24 hours in SU5402 had no effect on later bud development and detachment (n=100, control polyps; n=100, data not shown). None of the young polyps had developed beyond stage 5, when the SU5402 treatment was finished. Therefore, the decision to form a foot at the bud base takes time – the inhibitor needs to be present between stage 3 and 7 to show maximal effects.

Like SU5402, phosphorothioate (PT) antisense oligonucleotides specific for the *kringelchen* mRNA prevented detachment if electroporation was started at stage 3 (Fig. 5G,H). Despite the low survival rate after electroporation, the effect was specific. The data given in Fig. 5G is derived from three experiments with 20 polyps each, the 39 surviving polyps were evaluated. The abnormality is similar to the one described for SU5402, with the exception that the young polyps are slim and elongated (up to twice as long as normally), indicating that proportioning is affected as well (Fig. 5H). In both cases, the circular expression domain of *kringelchen* is distorted or missing (Fig. 5D-F,H) comparable with the above-described bud-head competition experiment. Two scrambled PT-oligonucleotides (n=60 each) had no effect.

Discussion

Cell migration, differentiation and tissue formation is often dependent on FGFR signalling, as shown in studies of limb bud,

trachea or heart development (Burke et al., 1998; Skaer, 1997). It has been unclear up to now whether FGF/FGFR signalling was invented at the level of triploblastic organisms (Coulier et al., 1997; Nagendra et al., 2001) or existed already in diploblastic coelenterates such as the cnidarian *Hydra*, which lacks organs but possesses tissue polarity. With the *Hydra* Kringelchen protein, we now add a new member to the family of FGF receptors, although this assignment is, at present, based on structural arguments rather than FGF binding.

Kringelchen sequence features suggest that it is an ancient FGFR

Characteristic features for FGFRs of triploblasts are: (1) an intracellular split tyrosine kinase domain and docking sites for downstream signalling cascades, (2) a single transmembrane domain and (3) three Ig-like loops (Ig I=D1 to Ig III=D3) in the extracellular, ligand-binding domain. Loops I and II are separated by an acidic stretch of amino acids, which carries a consensus sequence for CAM binding in vertebrates (Burke et al., 1998). The third Ig-like loop is optimized in vertebrates by alternative splicing for specific binding of one of the 22 FGF ligands (Ornitz and Itoh, 2001).

The putative intracellular domain of Kringelchen is highly conserved with a single transmembrane and a split kinase domain. Phylogenetic analysis of the latter places Kringelchen at the base of the FGFR. Docking sites relevant for downstream signalling are two SH2-binding domain consensus sequences, which are prerequisite to couple to the SH2 domain of PLCγ (Mohammadi et al., 1991) and a SH3-binding domain consensus for coupling to PI3-kinase. Thus, Kringelchen has the potential to activate the PLCγ/PKC cascade, which constitutes (together with the Ras/MAPK und PI3-kinase pathways) the main FGFR downstream signalling systems. Interesting in this respect is the observation that *Hydra* PKC2 (HvPKC2) (Hassel et al., 1998) is co-expressed with *kringelchen* early in the evaginating bud. No expression data are available for *Hydra* PLCγ yet (Koyanagi et al., 1998), but the preconditions for signalling through the Ras/MAPK pathway are given, as two *ras*-related genes have been identified by Bosch et al. (Bosch et al., 1995).

In the putative extracellular domain of Kringelchen, about 35% of the amino acids corresponding to Ig-like loops I, II and III of higher metazoan FGFR are identical to vertebrate and invertebrate receptors. The spacing and amino acid surrounding of four of the five extracellular cysteines in Kringelchen allow a clear assignment to the cysteines, which covalently clamp the D1 and D2 loops of higher metazoan FGFR (Fig. 1). As in other FGFRs, the region between D1 and D2 is acidic (pKa 4.41), but does not show a strong clustering of acidic amino acids.

The most conspicuous deviation from known FGFR is the lack of cysteines between the putative Ig-like loop II and the transmembrane domain, which excludes formation of a covalently linked D3. This feature is remarkable and raises the question if Kringelchen is able to bind FGF. D3 is crucial in vertebrates to confer specificity of FGF binding and together with D2 activates FGFR by FGF-induced dimerization. Point mutations in one or both cysteines of D3 cause ligand-independent dimerization (and constitutive activation), or generate FGFR with strongly reduced activity (Burke et al., 1998). The alignment in Fig. 1C reveals that the positions corresponding to the D3-clamping cysteines are taken in Kringelchen by Tyr249 and Phe324. Both amino acids are flanked by hydrophobic residues, which provide the structural precondition for a potential hydrophobic clamp. It is, thus, not excluded that even without cysteines a third Ig-like loop forms in *Hydra*. Interesting under the evolutionary aspect is that the Cys codons (T<u>G</u>T, T<u>G</u>C) can be generated by a single point mutation in position 2 of the codons for either Tyr (T<u>A</u>T, T<u>A</u>C) or Phe (T<u>T</u>T, T<u>T</u>C). With the transition from an ancient AT-rich genome, as found in *Hydra* (Galliot and Schummer, 1993), to the GC-rich genomes of higher organisms; a codon switch from Phe or Tyr to Cys in this critical position might have improved the performance of FGFR. A recently described platyhelminth protein that aligns with FGFR extracellular and transmembrane domains also lacks D3 (Cebria et al., 2002), but does not show a comparable amino acid arrangement.

The question, 'Do *Hydra* use FGF ligand(s) as signalling molecules?' cannot be answered yet, but a comparison of conserved residues identified in the crystal structure of vertebrate and invertebrate FGFR as interaction sites with FGF (Plotnikov et al., 1999; Nagendra et al., 2001) indicates a high level of conservation: of the 10 amino acids identified as binding partners for FGF2 in the D2 and D3 loops of the human FGFR1, Pro285, His286 and Asn345 are identical in *Hydra* (Pro 257, His258, Asn328); conserved exchanges are found in three more positions. The remainder is neither conserved in the protostome invertebrates nor in *Hydra*.

Consensus sequences that allow binding to other known extracellular FGFR interaction partners, namely heparan sulfates, which enhance FGF binding (Ornitz, 2000), or cell adhesion molecules (CAM), are not conserved in Kringelchen. Of the eight (mostly basic) amino acids identified in heparin binding, only one is present (Nagendra et al., 2001). An HAV motif, which is a hallmark of CAMs and found in the vertebrate FGFR adjacent to the acidic domain (Plotnikov et al., 1999), is missing in *Hydra* as well as in *Drosophila*. Thus, as deduced from its sequence, Kringelchen might bind FGF, but it is unlikely to function in a CAM-dependent manner, and it is highly questionable if Kringelchen binds heparan sulfates.

Kringelchen indicates changing positional values

It is striking that *kringelchen* RNA is detectable in regions only in which the positional value changes or from which adjacent tissue is organized: the bud tip is kringelchen-positive only as long as it organizes the axis (for a review, see Meinhardt, 1993); expression is switched off, when the positional value of a mature head is reached and tentacle buds form. The bud base is positive, as long as the positional value decreases to allow constriction and foot formation, but mature foot tissue is negative. Finally, the ring, which transiently persists in the parent after detachment, is positive until the cells have taken their normal positions. This expression pattern indicates that Kringelchen is involved in morphogenesis, but not in maintenance of structures. It fits well a recently published mathematical model for pattern formation in concentric rings (Berking, 2003), which predicts expression patterns in the growing bud similar to the *kringelchen* pattern.

Evidence for an evolutionarily conserved role of FGFR in boundary formation

The dynamic expression of *kringelchen* resembles FGFR

expression in the development of higher metazoa, where quick changes allow signalling through particular pairs of FGF/FGFR in a locally restricted manner (Ford-Periss et al., 2001). Comparison of budding to such morphogenetic processes reveals parallels to (1) branching morphogenesis (i.e. early budding) and (2) formation of boundaries (i.e. late budding, detachment). Relevant examples for branching morphogenesis are the formation of the tracheolar system in insects (Skaer, 1997) or of limbs in vertebrates (Gorivodsky and Lonai, 2003). While formation of the tracheolae requires the presence of FGF-secreting chemoattractant cells in the surrounding tissue, *Hydra* bud formation resembles mechanistically limb formation, where FGFR-based reciprocal inductive events control evagination from an existing axis, tissue movement, elongation and later, by coupling to downstream Wnt and PKC signalling, also differentiation and establishment of the complex limb bud pattern (for a review, see Wilkie et al., 2002). It is suggested that the *Hydra* Wnt homologue, *HyWnt*, and a PKC isoform, *HvPKC2*, form a synexpression group with *kringelchen* in the bud tip (Hobmayer et al., 2000; Hassel et al., 1998).

Despite these parallels, our inhibition experiments indicate that Kringelchen/FGFR signalling is, in contrast to the above-mentioned branching systems, neither involved in bud (i.e. branch) induction per se, nor the decisive factor for elongation and apical patterning. As *Hyβ-Cat* and *HyTcf* as members of the *Hydra* Wnt pathway are detected already in the bud induction phase, when *kringelchen* is still silent, it remains to be shown if the interconnection of signalling cascades is different in *Hydra* or if redundancy of signalling cascades ensures proper bud formation.

Our experiments indicate that the evolutionary conserved function of FGFR signalling, lies in boundary formation, which in the case of *Hydra* budding is necessary to set the stage for constriction and foot formation at the bud base. Boundary formation is an important FGFR function in vertebrates: FGFR control early patterning along the anteroposterior and dorsoventral axis of the brain, and are later essential to establish the midbrain-hindbrain boundary (Altmann and Brivanlou, 2001). A recent report further corroborates this hypothesis, because the transient knockout of Nou Darake in the platyhelminth *Dugesia* led to a posterior shift of brain structures indicating a defective boundary (Cebria et al., 2002). Nou Darake is a transmembrane protein, with high similarity to the extracellular domain of FGFR, but lacks Ig-like loop III and a tyrosine kinase domain.

The mechanism by which Kringelchen inhibition prevents detachment is very likely to be complex. In SU5402-treated buds, the normal *kringelchen* expression zone is distorted or missing, thus the inhibitor cannot directly interfere with protein at the bud base and thereby inhibit detachment. RT-PCR detects *kringelchen* expression all along the body column at a very low level, and as FGFRs are often upregulated by autocatalytic loops and then either act in a morphogen- or a threshold-like mode (Hajihosseini et al., 2004), our working hypothesis is that SU5402 inhibits Kringelchen upregulation by blocking an autocatalytic loop close to the bud base. The crucial phase for this inhibition is between bud stage 3 and 7.

These conclusions are, of course, valid only if SU5402 inhibits Kringelchen as specifically as it inhibits other FGFRs, which has to be shown experimentally in the future. In previous reports, non-detaching buds resulted from treatment with more nonspecific protein (tyrosine) kinase inhibitors (Perez and Berking, 1994; Fabila et al., 2002) or with lithium ions (Hassel and Berking, 1990). Strong arguments can now be made in favour of an FGFR-specific effect. First, we obtained almost identical abnormalites using SU5402 and *kringelchen* antisense inhibition; second, SU5402 is described as highly specific for FGFR with no activity in insulin or EGFR, and only weak reactivity with PDGFR in sixfold higher concentrations as used in this study. Moreover, seven out of eight residues identified as important for SU5402-FGFR binding (Mohammadi et al., 1997) are identical in *Hydra*. Non-FGFR tyrosine kinases lack at least three (VEGFR) out of these eight crucial residues. And last but not least, Southern, northern blots and the *Hydra* EST databases do not provide evidence for another, closely related *Hydra* FGFR, which might be the target for inhibition. We therefore conclude that the described Kringelchen inhibition effects are specific and identify a role in boundary formation as evolutionary conserved function of FGFR.

In the near future, it will be very interesting to investigate, which ligand(s) activate Kringelchen with its peculiar structural properties. Although it seemed for a long time as though peptides might function in cnidaria like the complex growth factors in triploblasts (for a review, see Bosch and Fujisawa, 2001), data are accumulating from cnidarian EST projects that *Hydra*, like all higher evolved metazoa, possesses growth factor homologues and their potential receptors. It will be interesting to elucidate the relationship of peptide and growth factor signalling in cnidarian morphogenesis.

We thank Stefan Berking and Renate Renkawitz-Pohl for critical reading of the manuscript. This work was supported by the DFG, grants Ha 1732/4, 1732/8 and funding of S.S. by the Graduiertenkolleg ‘Signalling systems and gene expression in developmental model organisms’.

References

Affolter, M. and Shilo, B. Z. (2000). Genetic control of branching morphogenesis during *Drosophila* tracheal development. *Curr. Opin. Cell Biol.* **12**, 731-735.

Altmann, C. R. and Brivanlou, A. H. (2001). Neural patterning in the vertebrate embryo. *Int. Rev. Cytol.* **203**, 447-482.

Berking, S. (2003). A model for budding in hydra: pattern formation in concentric rings. *J. Theor. Biol.* **222**, 37-52.

Borland, C. Z., Schutzman, J. L. and Stern, M. J. (2001). Fibroblast growth factor signalling in *Caenorhabditis elegans*. *BioEssays* **23**, 1120-1130.

Bosch, T. C. and Fujisawa, T. (2001). Polyps, peptides and patterning. *BioEssays* **23**, 420-427.

Bosch, T. C., Benitez, E., Gellner, K., Praetzel, G. and Salgado, L. M. (1995). Cloning of a ras-related gene from *Hydra* which responds to head-specific signals. *Gene* **167**, 191-195.

Burke, D., Wilkes, D., Blundell, T. L. and Malcolm, S. (1998). Fibroblast growth factor receptors: lessons from a gene. *TIBS* **23**, 59-61.

Brysch, W. and Schlingensiepen, K. H. (1994). Design and application of antisense oligonucleotides in cell culture, in vivo, and as therapeutic agents. *Cell. Mol. Neurobiol.* **14**, 557-568.

Cebria, F., Kobayashi, C., Umesono, Y., Nakazawa, M., Mineta, K., Ikeo, K., Gojobori, T., Itoh, M., Taira, M., Alvarado, A. S. and Agata, K. (2002). FGFR-related gene *nou-darake* restricts brain tissue to the head of planarians. *Nature* **424**, 743-748.

Coulier, F., Pontarotti, P., Roubin, R., Hartung, H., Goldfarb, M. and Birnbaum, D. (1997). Of worms and men: an evolutionary perspective on the fibroblast growth factor (FGF) and FGF receptor family. *J. Mol. Evol.* **44**, 43-56.

Fabila, Y., Navarro, L., Fujisawa, T., Bode, H. R. and Salgado, L. M. (2002). Selective inhibition of protein kinases blocks the formation of a new axis, the beginning of budding in *Hydra*. *Mech. Dev.* **119**, 157-164.

Ford-Periss, M., Abud, H. and Murphy, M. (2001). Fibroblast growth factors in the developing central nervous system. *Clin. Exp. Pharmacol. Physiol.* **28**, 493-503.

Galliot, B. and Schummer, M. (1993). 'Guessmer' screening strategy applied to species with AT-rich-coding sequences. *TIG* **9**, 3-4.

Gawantka, V., Pollet, N., Delius, H., Vingron, M., Pfister, R., Nitsch, R., Blumenstok, C. and Niehrs, C. (1998). Gene expression screening in *Xenopus* identifies molecular pathways, predicts gene function and provides a global view of embryonic patterning. *Mech. Dev.* **77**, 95-141.

Goridowski, M. and Lonai, P. (2003). Novel roles of FGFR2 in AER differentiation and positioning of the dorsoventral limb interface. *Development* **130**, 5471-5479.

Grassot, J., Mouchiroud, G. and Perrière, G. (2003). RTKdb: database of receptor tyrosine kinase. *Nucleic Acids Res.* **31**, 353-358.

Grens, A., Gee, L., Fisher, D. A. and Bode, H. R. (1996). CnNK-2, an NK-2 homeobox gene, has a role in patterning the basal end of the axis in hydra. *Dev. Biol*. **180**, 473-488.

Hajihosseini, M. K., Lalioti, M. D., Arthaud, S., Burgar, H. R., Brown, J. M., Twigg, S. R., Wilkie, A. O. and Heath, J. K. (2004). Skeletal development is regulated by fibroblast growth factor receptor 1 signalling dynamics. *Development* **131**, 325-335.

Hassel, M. and Berking, S. (1990). Lithium ions interfere with pattern control in *Hydra* vulgaris. *Roux's Arch. Dev. Biol*. **198**, 382-388.

Hassel, M., Albert, K. and Hofheinz, S. (1993). Pattern formation in *Hydra vulgaris* is controlled by lithium-sensitive processes. *Dev. Biol.* **156**, 362-371.

Hassel, M., Bridge, D. M., Stover, N. A., Kleinholz, H. and Steele, R. E. (1998). The level of expression of a protein kinase C gene may be an important component of the patterning process in *Hydra*. *Dev. Genes Evol.* **207**, 502-514.

Hobmayer, B., Rentzsch, F., Kuhn, K., Happel, C. M., Cramer von Laue, C., Snyder, P., Rothbaecher, U. and Holstein, T. W. (2000). WNT signalling molecules act in axis formation in the diploblastic metazoan *Hydra*. *Nature* **407**, 186-189.

Hoffmeister, S. A. H. and Schaller, C. H. (1985). A new biochemical marker for foot-specific cell differentiation in *Hydra. Roux's Arch. Dev. Biol.* **194**, 453-461.

Koyanagi, M., Ono, K., Suga, H., Iwabe, N. and Miyata, T. (1998). Phospholipase C cDNAs from sponge and hydra: antiquity of genes involved in the inositol phospholipid signaling pathway. *FEBS Lett.* **439**, 66-70.

Kouhara, H., Hadari, Y. R., Spivak-Kroizman, T., Schilling, J., Bar-Sagi, D., Lax, I. and Schlessinger, J. (1997). A lipid-anchored Grb2-binding protein that links FGF-receptor activation to the Ras/MAPK signaling pathway. *Cell* **89**, 693-702.

Lohmann, J. U., Endl, I. and Bosch, T. C. (1999). Silencing of developmental genes in *Hydra*. *Dev. Biol.* **214**, 211-214.

Meinhardt, H. (1993). A model for pattern formation of hypostome, tentacles, and foot in hydra: how to form structures close to each other, how to form them in a distance. *Dev. Biol*. **157**, 321-333.

Mohammadi, M., Honegger, A. M., Rotin, D., Fischer, R., Bellot, F., Li, W., Dionne, C. A., Jaye, M., Rubinstein, M. and Schlessinger, J. (1991). A tyrosine-phosphorylated carboxy-terminal peptide of the fibroblast growth factor receptor (Flg) is a binding site for the SH2 domain of phospholipase C-gamma. *Mol. Cell. Biol*. **11**, 5068-5078.

Mohammadi, M., McMahon, G., Sun, L., Tang, C., Hirth, P., Yeh, B. K., Hubbard, S. R. and Schlessinger, J. (1997). Structures of the tyrosine kinase domain of fibroblast growth factor receptor in complex with inhibitors. *Science* **276**, 955-960.

Nagendra, H. G., Harrington, A. E., Harmer, N. J., Pellegrini, L., Blundell, T. L. and Burke, D. F. (2001). Sequence analyses and comparative modeling of fly and worm fibroblast growth factor receptors indicate that the determinants for FGF and heparin binding are retained in evolution. *FEBS Lett.* **501**, 51-58.

Ogawa, K., Kobayashi, C., Hayashi, T., Orii, H., Watanabe, K. and Agata, K. (2002). Planarian fibroblast growth factor receptor homolog expressed in stem cells and cephalic ganglions. *Dev. Growth Differ.* **44**, 191-204.

Ornitz, D. M. (2000). FGFs, heparan sulfate and FGFRs: complex interactions essential for development. *BioEssays* **22**, 108-112.

Ornitz, D. M. and Itoh, N. (2001). Fibroblast growth factors. *Genome Biol.* **2**, REVIEWS 3005.

Otto, J. J. and Campbell, R. D. (1977). Budding in *Hydra attenuata*: bud Stages and Fate Map. *J. Exp. Zool.* **200**, 417-428.

Perez, F. and Berking, S. (1994). Protein kinase modulators interfere with bud formation in *Hydra vulgaris*. *Roux's Arch. Dev. Biol.* **203**, 284-289.

Plotnikov, A. N., Schlessinger, J., Hubbard, S. R. and Mohammadi, M. (1999). Structural basis for FGF receptor dimerization and activation. *Cell* **98**, 641-650.

Skaer, H. (1997). Morphogenesis: FGF branches out. *Curr. Biol.* **7**, R238-R241.

Sleeman, M., Fraser, J., McDonald, M., Yuan, S., White, D., Grandison, P., Kumble, K., Watson, J. D., Murison, J. G. (2001). Identification of a new fibroblast growth factor receptor, FGFR5. *Gene* **271**, 171-182.

Stover, N. A. and Steele, R. E. (2001). Trans-spliced leader addition to mRNAs in a cnidarian. *Proc. Natl. Acad. Sci. USA* **98**, 5693-5698.

Tardent, P. (1972). Experimente zum Knospungsbildungsprozess von *Hydra attenuata* (PALL.). *Rev. Suisse Zool.* **79**, 355-375.

Trueb, B., Zhuang, L., Taeschler, S. and Wiedemann, M. (2003). Characterization of FGFRL1, a novel fibroblast growth factor (FGF) receptor preferentially expressed in skeletal tissues. *J. Biol. Chem.* **278**, 33857-33865.

Wilkie, A. O. M., Patey, S. J., Kan, S. and van den Ouweland, A. M. W. (2002). FGFs, their receptors, and human limb malformations: clinical and molecular correlations. *Am. J. Med. Genet*. **112**, 266-278.

Yamaguchi, T. P. and Rossant, J. (1995). Fibroblast growth factors in mammalian development. *Curr. Opin. Genet. Dev.* **5**, 485-491.