

# Sweet Tooth, a Novel Receptor Protein-tyrosine Kinase with C-type Lectin-like Extracellular Domains\*

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**A gene encoding a novel type of receptor protein-tyrosine kinase was identified in *Hydra vulgaris*. The extracellular portion of this receptor (which we have named *Sweet Tooth*) contains four C-type lectin-like domains (CTLDs). Comparison of the sequences of these domains with the sequences of the carbohydrate recognition domains of various vertebrate C-type lectins shows that *Sweet Tooth* CTLD1 and CTLD4 have amino acids in common with those shown to be involved in carbohydrate binding by the lectins. Comparison of sequences encoding CTLD1 from the *Sweet Tooth* genes from different species of *Hydra* shows variation in some of the conserved residues that participate in carbohydrate binding in C-type lectins. The *Sweet Tooth* gene is expressed widely in the *Hydra* polyp, and expression is particularly high in the endoderm of the tentacles. Treatment of polyps with peptides corresponding to sequences in the *Sweet Tooth* CTLDs results in the disintegration of the animal. These same peptides do not block adhesion or morphogenesis of *Hydra* cell aggregates.**

Receptor protein-tyrosine kinases (RTKs)<sup>1</sup> are used to transduce the signals required for a variety of developmental and physiological processes in multicellular animals (1). No RTKs have been identified in unicellular organisms, and the yeast genome lacks sequences encoding protein-tyrosine kinases (2). It is likely, therefore, that the evolution of RTKs was a critical event in the formation of multicellular animals. Because the bulk of characterized RTKs are from a small number of triploblastic animal species, we have a relatively restricted view of the evolutionary history of these molecules. It is by no means clear that the classes of RTKs identified to date in higher animals contain examples of all of the types of RTKs that multicellular cellular animals have evolved. Sponges and cni-

darians contain RTKs, indicating that such receptors existed in the common ancestor of all extant metazoans. A homologue of the vertebrate insulin receptor is present in the cnidarian *Hydra vulgaris* (3), and a gene encoding an RTK containing Ig-like domains in its extracellular portion has been identified in a sponge (4, 5). These findings establish the continuity of two types of RTKs from the earliest stages of metazoan evolution through to vertebrates.

We know even less about the evolution of ligands for RTKs than we know about the evolution of the receptors. Only a small number of RTK ligands from invertebrate phyla have been characterized. All of the RTK ligands that have been identified to date in both invertebrates and vertebrates are proteins, although in some cases RTKs have been shown to interact in a homophilic manner (6) and thus appear to lack a separate ligand molecule. Notably lacking are examples of carbohydrates as RTK ligands. Given the diversity of carbohydrates displayed on the surfaces of animal cells, it is perhaps surprising that carbohydrates appear not to have been exploited as ligands for RTK-mediated signaling pathways. In searching for RTKs that play roles in developmental processes in *Hydra*, we have identified a gene (*Sweet Tooth*) encoding a novel RTK with an extracellular portion that contains four C-type lectin-like domains (CTLDs). If the CTLDs of *Sweet Tooth* bind carbohydrate, this receptor would be the first example of an RTK that is activated by carbohydrate ligands. Potential processes in which such a receptor could be involved include cell adhesion, morphogenesis, and recognition of foreign cells. Peptide blocking experiments with *Hydra* polyps suggest that *Sweet Tooth* is not required for cell adhesion or morphogenesis. The possibility that *Sweet Tooth* acts as a pattern recognition receptor for foreign cells and thus may be a component of an immune system in *Hydra* cannot be ruled out.

## EXPERIMENTAL PROCEDURES

**Hydra Culture**—The various species and strains of *Hydra* were obtained from the laboratory of Hans Bode (University of California, Irvine, CA) and maintained according to standard methods (7). The 105 strain of *H. magnipapillata* was originally from the laboratory of Tsutomu Sugiyama. The UCI and Basel strains of *H. vulgaris*, and the Swiss *H. oligactis* strain were originally from the laboratory of Pierre Tardent.

**Isolation of *Sweet Tooth* cDNA Clones**—Amplification and cloning of cDNA fragments from genes encoding protein-tyrosine kinases were carried out essentially as described previously (3, 8). Clones were screened by sequencing using only the dideoxy-T stop reaction mixture (T-tracking). One of the amplified DNA fragments, designated HTK27, was used to isolate several clones from a  $\lambda$ gt11 cDNA library prepared from adult polyps of *H. vulgaris* (9). Sequence analysis of these clones revealed that they were incomplete, consisting only of the portion of the cDNA encoding the kinase domain. A fragment from the 5' end of one of the HTK27 clones was then used to screen a  $\lambda$ ZAPII cDNA library made from whole adult polyp poly(A)<sup>+</sup> RNA (10). From this screen we obtained clones from a new gene related to HTK27, which we termed *Sweet Tooth*. Rescreening of the  $\lambda$ ZAPII cDNA library with a *Sweet Tooth* probe yielded clones that in aggregate included the entire *Sweet*

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) L22612, AF129527, AF129528, and AF129529.

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<sup>1</sup> The abbreviations used are: RTK, receptor protein-tyrosine kinase; CTLD, C-type lectin-like domain; CRD, carbohydrate recognition domain; TFA, trifluoroacetic acid; HPLC, high-pressure liquid chromatography; MBP-A, mannose-binding protein A.

*Tooth* coding sequence. Library screens and DNA sequencing were carried out essentially as described previously (3).

**RNA Hybridization**—Poly(A)<sup>+</sup> RNA was extracted from adult *H. vulgaris* polyyps using the Amersham Pharmacia Biotech Quick Prep mRNA kit. Electrophoresis of the RNA in a formaldehyde-agarose gel, transfer to a nylon filter, and hybridization were carried out essentially as described previously (11).

**Protein Expression in Yeast**—A fragment encoding the kinase domain and carboxyl-terminal tail of *Sweet Tooth* (amino acids 940–1348) was amplified from one of the cDNA clones using primers that included a *SalI* cleavage site at the 5' end of the 5' primer and an *XbaI* cleavage site at the 5' end of the 3' primer. The 5' primer also included an ATG codon, the eight nucleotides upstream of the ATG in the GAL4 gene (5'-CCTGAAAGATG-3'), and the following *Sweet Tooth* sequence: 5'-T-ATCAAATGGGGTGTGATG-3'. The 3' primer contained the following *Sweet Tooth* sequence: 5'-CGGCACGAGCGGCACGAG-3'. This sequence is located in the 3' untranslated sequence of the gene. Amplification of the entire coding region of the *Src2* gene from *Xenopus laevis* (12) was performed using gene-specific primers with *BglII* and *XbaI* cleavage sites attached to the 5' and 3' primers, respectively. The amplified fragments were isolated from the reaction mixtures with QIAEX particles (Qiagen), cleaved with the appropriate restriction enzymes, and purified by agarose gel electrophoresis. Fragments were ligated into the galactose-inducible yeast expression vector pRS316-GAL1 (13), which had been cleaved with either *SalI* and *XbaI* (for the *Sweet Tooth* fragment) or *BamHI* and *XbaI* (for the *Src2* fragment). Strain W303 of *Saccharomyces cerevisiae* was transformed with plasmid DNA using the lithium acetate method (14). Transformants were selected on minimal medium lacking uracil. For expression of the *Sweet Tooth* and *Src2* proteins, plasmid-containing yeast cells were grown at 30 °C in uracil-minus minimal medium plus glucose overnight and then diluted 1:250 in uracil-minus minimal medium plus galactose and grown for an additional 20 h.

Cells were harvested by centrifugation, and proteins were extracted by alkaline lysis (15). Protein concentrations were determined using the BCA protein assay reagent kit (Pierce). Equal amounts of protein were fractionated by SDS polyacrylamide gel electrophoresis and transferred to an Immobilon-P filter (Millipore) using a Bio-Rad Trans-Blot SD semidry electrophoretic transfer cell. The filter was blocked in 5% bovine serum albumin for 1 h at room temperature and incubated with 4G10 anti-phosphotyrosine antibody (Upstate Biotechnology) in Tris-buffered saline plus 0.05% Tween 20 at a dilution of 1:13,000. The filter was then washed at room temperature five times for 5 min each in Tris-buffered saline plus 0.05% Tween 20. After incubation for 1 h at room temperature with a horseradish peroxidase-conjugated goat antimouse IgG antibody (Transduction Laboratories) diluted 1:25,000 in Tris-buffered saline plus 0.05% Tween 20, the filter was washed at room temperature four times for 5 min each in Tris-buffered saline plus 0.05% Tween 20. Bound antibody was detected with the SuperSignal chemiluminescent substrate for Western blotting (Pierce).

**Amplification of CTLD1 Genomic Sequences**—Total DNA was isolated from *H. vulgaris* (Basel and UCI strains), *H. magnipapillata* (105 strain), and *H. oligactis* (Swiss strain) using a rapid extraction method (16). PCR primers were designed to amplify the DNA sequence encoding CTLD1. The 5' primer sequence was 5'-TCCGGATCCGTAAGCAATAGCTGTGACA-3' and included a *BamHI* cleavage site at the 5' end (underlined). The 3' primer was 5'-GCAAGCTTTTCTAATTTTACAAATAAATCC-3' and included a *HindIII* cleavage site at the 5' end (underlined). These primers were used at a concentration of 100 pmol/μl with 100 ng of *Hydra* DNA as a template in a 100-μl reaction containing 1 × buffer (supplied with the *Taq* DNA polymerase), 4 mM MgCl<sub>2</sub>, and 2.5 units of *Taq* DNA polymerase (Promega). The amplified DNA fragment was isolated by electrophoresis in a 3% NuSieve (FMC Bioproducts) agarose gel in Tris borate-EDTA buffer and extraction from the gel using the Qiaex gel extraction kit (Qiagen). The isolated fragment was cut with the restriction enzymes *BamHI* and *HindIII*, purified again by electrophoresis, and then subcloned into pBluescript KS+ (48) that was cut with the same restriction enzymes.

**In Situ Hybridization**—Whole-mount *in situ* hybridization to *H. vulgaris* polyyps was carried out essentially as described previously (17, 18). A mixture of three digoxigenin-labeled RNA probes was used. Probes were made from cDNA fragments encoding CTLDs 1, 3, and 4. To generate these fragments we amplified with the following primers: CTLD1 (see above); CTLD3, 5' primer (5'-GCGGATCCAAACAATAAATAGCTGTAG-3') and 3' primer, (5'-GCAAGCTTAACTTACAATAAATAGCTG-3'), and CTLD4, 5' primer (5'-GCGGATCCCACTAATGATTGCGCTG-3') and 3' primer (5'-TCCAAGCTTGCTTAGGTAAAGTTCTTC-3'). Each primer pair contained a *BamHI* cleavage site in the 5'

primer and a *HindIII* cleavage site in the 3' primer.

DNA amplification was performed as described above for genomic DNAs, except cDNA clones were used as the templates. The amplified DNA fragments were cut with *BamHI* and *HindIII*, purified using the Qiaex gel extraction kit (Qiagen), and then subcloned into the pBlue-scriptII KS+ vector cleaved with *BamHI* and *HindIII*. *In vitro* transcription reactions using T7 and T3 polymerases (Promega), digoxigenin-UTP (Roche Molecular Biochemicals), and linearized plasmid templates to yield sense and antisense probes were carried out according to standard methods.

After *in situ* hybridization, polyyps were permanently mounted on microscope slides using Euparal (Asco Laboratories). Photographs were taken with an Olympus Vanox microscope using Nomarski optics and Eastman Kodak Co. Ektachrome 160T film.

**Synthetic Peptide Treatment**—Synthetic peptides were synthesized that corresponded to a conserved eight-amino acid sequence in each of the four CTLDs of *Sweet Tooth* (see Fig. 1B). In addition, a control peptide was synthesized that was a scrambled version of the CTLD1 peptide. Peptide synthesis was performed by Quality Controlled Biochemicals, Inc. All peptides were amidated on the carboxyl terminus. The amino acid sequences of the peptides are as follows: CTLD1, NYWIGLND-NH<sub>2</sub>; CTLD2, FFWIGLNY-NH<sub>2</sub>; CTLD3, NYWIGLTD-NH<sub>2</sub>; CTLD4, KYWIGLNK-NH<sub>2</sub>; and scrambled, LINGDYNW-NH<sub>2</sub>. The peptides were received as acetate salts. Two milligrams of each peptide were resuspended in 0.1% trifluoroacetic acid (TFA) in water for purification using HPLC on a 10-mm Vydac C4 semipreparative column. The peptides were eluted with a gradient starting with 90 parts 0.1% TFA in water and 10 parts 0.09% TFA in acetonitrile to 40 parts 0.1% TFA in water and 60 parts 0.09% TFA in acetonitrile. The flow rate was 3 ml/min. Peptide fractions were detected by UV absorbance at 280 nm. The major absorbance peak samples were pooled, shell frozen, and lyophilized to dryness. Samples were resuspended in 1 ml of *Hydra* medium and centrifuged at 1400 rpm in a microcentrifuge to remove insoluble material. The supernatant was transferred to a new tube, and its absorbance at 280 nm was measured. The concentration of each peptide was determined from the absorbance using calculated molar extinction coefficients. Mass spectroscopy of the collected fraction for the CTLD1 peptide was carried out to verify that the HPLC purification was performing as expected. Purification was successful for the peptides corresponding to CTLDs 1, 3, and 4. The peptide for CTLD2 was insoluble in 0.1% TFA in water and therefore could not be used for experiments.

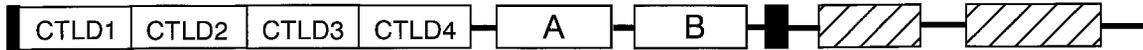
The peptides were used at a concentration of 100 μM in *Hydra* medium at 18 °C. Exposure of intact or regenerating animals to peptides was carried out on groups of 10 animals in 1 ml of medium in the well of a 24-well microtiter plate. A mixture of the three peptides was tested as well as individual peptides. For control experiments either a scrambled version of the peptide for CTLD1 in *Hydra* medium or *Hydra* medium alone was used. Fresh peptide-containing medium was added daily during the course of the experiment. Animals were fed 2 days before the start of peptide treatment but were not fed during peptide treatment.

Aggregates of cells of the UCI strain of *H. vulgaris* were prepared according to standard methods (19, 20). Peptides were added both before and immediately after centrifugation of the cells to form the aggregate. If the peptide was added before centrifugation, a 1-h incubation at 18 °C with constant mild agitation was performed before centrifugation. Incubation was continuous after centrifugation, with three aggregates per well per 100 μl of solution in 96-well microtiter plates at 18 °C. Fresh peptide-containing medium was added 8, 24, and 32 h after aggregate formation.

## RESULTS

**The *Sweet Tooth* Gene Encodes a Receptor Protein-tyrosine Kinase with Extracellular C-type Lectin-like Domains**—The organization of the protein encoded by the *Sweet Tooth* gene is shown schematically in Fig. 1A. The protein contains a predicted signal peptide sequence at the amino terminus, a predicted transmembrane sequence, and a typical protein-tyrosine kinase catalytic domain. Phylogenetic comparison of the *Sweet Tooth* catalytic domain sequence with sequences in the data bases indicates that it is most closely related to members of the subfamily of protein-tyrosine kinases that includes Ret, the fibroblast growth factor receptors, and the platelet-derived growth factor receptors and their relatives (data not shown). Comparison of the sequence of the extracellular region of the

A



B

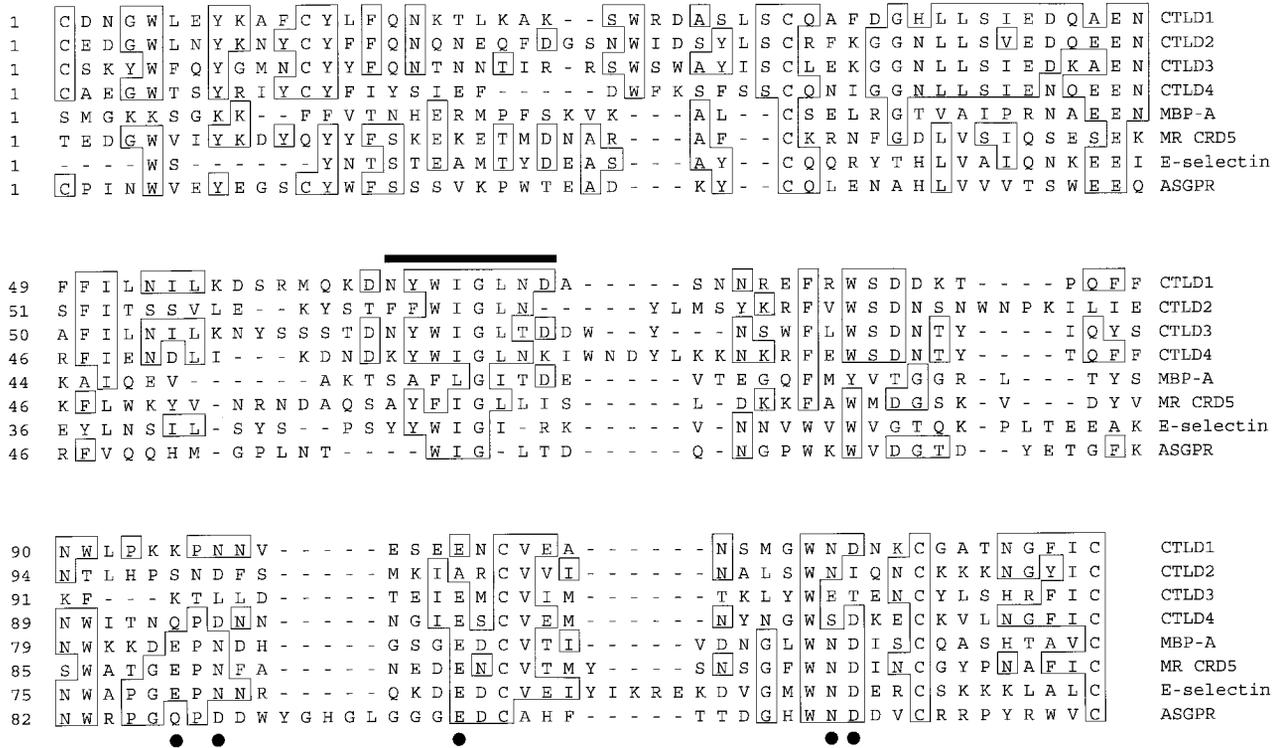


FIG. 1. A, domain structure of the predicted Sweet Tooth protein from *H. vulgaris* (UCI strain). The signal peptide and transmembrane domain are indicated by black boxes. The two portions of the kinase domain are indicated by cross-hatched boxes. The four putative carbohydrate recognition domains are indicated by the boxes labeled CTL D1–CTL D4. The two copies of the repeated sequence in the extracellular domain are indicated by boxes A and B. B, amino acid sequences of the Sweet Tooth CTLDs aligned with CRD sequences from vertebrate C-type lectins. Amino acids present in a majority of the sequences are boxed. The abbreviations and references for the vertebrate sequences are as follows: MR CRD5, CRD5 from the human macrophage mannose receptor; MBP-A, rat mannose-binding protein A (45); ASGPR, rat asialoglycoprotein receptor (46); and E-selectin (47). The alignment was carried out using CLUSTAL as implemented by the Megalign program of the LASERGENE package (DNASTAR). Dots under the sequences indicate the positions of residues that have been shown to participate in the binding of the site 2 calcium ion in MBP-A (22). The bar indicates the sequences contained in the synthetic peptides used in the blocking experiments.

Sweet Tooth protein with the data bases yielded the unexpected finding that it contains four CTLDs (Fig. 1A, CTL D1–CTL D4), a domain type that has not been found in any other RTK. In addition, the extracellular portion of the protein contains two divergent copies (Fig. 1A, A and B) of a sequence with no significant similarity to any sequences in the data base. An alignment of the amino acid sequences of the predicted CTLDs from Sweet Tooth with the CTLDs from various vertebrate C-type lectins is shown in Fig. 1B. Phylogenetic analyses with the extracellular and kinase domain sequences of Sweet Tooth do not allow conclusions to be made regarding the evolutionary origin of this gene. Construction of phylogenetic trees using a variety of CTL D sequences shows that the four CTLDs of Sweet Tooth are more closely related to each other than to other CTLDs (data not shown). Although the sequence of the kinase domain of Sweet Tooth is more closely related to those of the Ret, fibroblast growth factor, and platelet-derived growth factor RTK families than to other RTK kinase domains, phylogenetic analysis does not support a close evolutionary relationship between Sweet Tooth and the members of these families (data not shown). The RTKs of *C. elegans*, all of which are now known because of completion of the genome sequence, do not include any that contain CTLDs (21).

C-type lectin domains contain a characteristic pattern of cysteine residues as well as additional highly conserved resi-

dues (22). All of the cysteines are conserved in the four CTLDs of Sweet Tooth as are virtually all of the conserved residues (Fig. 1B). Of particular interest are residues that have been shown to be involved in coordinated binding of calcium and mannose in mannose-binding protein A (MBP-A; Ref. 22). This binding involves the 5 residues indicated by dots in Fig. 1B. Four of these 5 residues, as well as the proline separating the first two of the residues (EPN), are conserved in CTL D1 of Sweet Tooth. The replacement of glutamic acid by lysine at the first of these positions in CTL D1 of Sweet Tooth replaces a negative, calcium binding carboxyl group with a positively charged amino group. This would preclude an interaction of this residue with calcium. Whether it is sufficient to exclude calcium binding and/or carbohydrate binding by CTL D1 is not known. CTLDs 2 and 3 show significant deviations at the five calcium and carbohydrate binding positions and would thus seem less likely to bind carbohydrate in a calcium-dependent manner. CTL D4 contains the sequence QPD instead of EPN at positions 94–96. This sequence occurs in the asialoglycoprotein receptor, which recognizes galactose, and has been shown to alter the specificity of MBP-A to galactose when it replaces the EPN sequence in MBP-A (23, 24). In addition, CTL D4 also contains the other residues involved in calcium and carbohydrate binding with the exception of a substitution of serine for asparagine at position 113. This substitution would potentially

provide the serine hydroxyl group for calcium coordination. Taken together, the sequence data suggest that CTLD1 and CTLD4 are good candidates for binding carbohydrate and that CTLD4 may recognize galactose.

Hybridization of poly(A)<sup>+</sup> RNA from adult *Hydra* polyps with a probe mixture specific for the sequence encoding the extracellular portion of *Sweet Tooth* detects a single 4.4-kb RNA species (Fig. 2). Assuming the presence of a poly(A) tail of 100–200 nucleotides, the RNA is the size expected from the sequence of the cDNA (4202 nucleotides).

**Evolution of a *Sweet Tooth* CTLD**—To explore the evolution of the *Sweet Tooth* gene, we amplified the sequences encoding CTLD1 from genomic DNA from another strain of *H. vulgaris* (Basel strain) and from two other species of *Hydra* (*H. magni-*

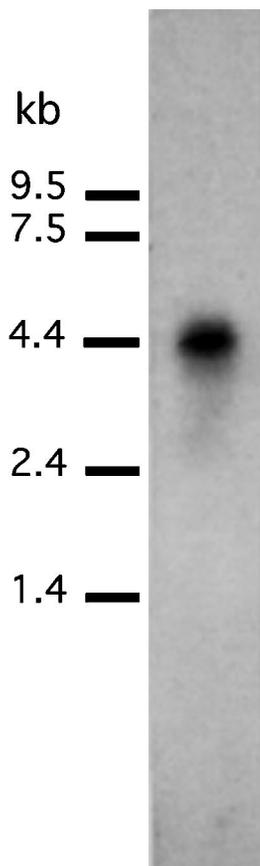


FIG. 2. Hybridization of *Sweet Tooth* RNA. Poly(A)<sup>+</sup> RNA (2  $\mu$ g) from adult *H. vulgaris* polyps was probed with a mixture of cDNA fragments encoding the extracellular portion of *Sweet Tooth*. The positions of RNA size markers (Life Technologies) that were electrophoresed in the same gel with the *Hydra* RNA are indicated.

FIG. 3. Predicted amino acid sequences of CTLD from the *Sweet Tooth* genes of *H. magnipapillata*, *H. oligactis*, and the UCI and Basel strains of *H. vulgaris*. Amino acids identical to those in the sequence of the UCI strain are boxed. The amino-terminal sequence VSNSCD and the carboxyl-terminal sequence GFICKIRK were encoded by the primers used for amplification.

1	V S N S C D N G W L E Y K A F C Y L F Q N K T L K A K S W R	<i>H. vulgaris</i> (UCI)
1	V S N S C D N G W L E Y K A F C Y L F Q N K T L K A K I N W R	<i>H. vulgaris</i> (Basel)
1	V S N S C D N G W L E Y K T F C Y F F Q N K T L Q A K N W R	<i>H. magnipapillata</i>
1	V S N S C D N G W L G Y G S Y C Y F F Q N K T L Q G T S W R	<i>H. oligactis</i>
31	D A S L S C Q A F D G H L L S I E D Q A E N F F I L N I L K	<i>H. vulgaris</i> (UCI)
31	D A S L S C Q A F D G H L L S I E D Q A E N F F I L N I L K	<i>H. vulgaris</i> (Basel)
31	D A S L S C Q A L F G H L L S I E D Q A E N F F I L N F L K	<i>H. magnipapillata</i>
31	D A S L S C Q V M G A H L L S I M D Q A E N I F I T N T L K	<i>H. oligactis</i>
61	D S R M Q K D N Y W I G L N D A S N N R E F R W S D D K T P	<i>H. vulgaris</i> (UCI)
61	D S R M Q K D N Y W I G L N D A S N N R E F R W S D E K T P	<i>H. vulgaris</i> (Basel)
61	D S S M Q Q D N Y W I G L N D A S N N R E F R W S D D K I P	<i>H. magnipapillata</i>
61	D D S R K K D N Y W I G F N D A Y N N R E F L W L D N T Y P	<i>H. oligactis</i>
91	Q F F N W L P K K P N N V E S E E N C V E A N S M G W N D N	<i>H. vulgaris</i> (UCI)
91	L F F N W L P K K P N N V E S E E N C V E A N S M G W N D N	<i>H. vulgaris</i> (Basel)
91	Q F F N W L P K K P N N D Q S E Q N C V E I T N S M G W N D E	<i>H. magnipapillata</i>
91	Q F Y D W L K G R P D N Y D S S E N C A V L D S I G W N D K	<i>H. oligactis</i>
121	K C G A T N G F I C K I R K	<i>H. vulgaris</i> (UCI)
121	K C G A T N G F I C K I R K	<i>H. vulgaris</i> (Basel)
121	N C D A T N G F I C K I R K	<i>H. magnipapillata</i>
121	D C S D T F G F I C K I R K	<i>H. oligactis</i>

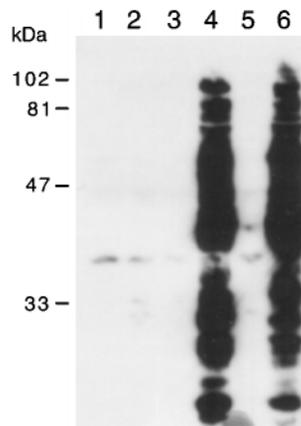
*papillata* and *H. oligactis*). In all three of the amplified fragments an intron was located at a position identical to that in the CTLD1 sequence from the UCI strain of *H. vulgaris* (data not shown), supporting the conclusion that the fragments were from the *Sweet Tooth* gene in each case. An alignment of the predicted amino acid sequences for CTLD1 from the UCI and Basel strains of *H. vulgaris* and *H. magnipapillata* and *H. oligactis* is shown in Fig. 3. As expected from previous phylogenetic analyses of these species,<sup>2</sup> the *H. magnipapillata* sequence is more closely related to the *H. vulgaris* sequences than is the *H. oligactis* sequence. Of particular interest is the fact that the sequence that is expected to be involved in carbohydrate selectivity in CTLD1 of *H. vulgaris* (KPN) contains potentially significant changes in *H. oligactis* (RPD). This finding suggests that if CTLD1 binds carbohydrate, it may bind a different carbohydrate in *H. oligactis* than in *H. vulgaris* and *H. magnipapillata*. We were unable to amplify DNA from *H. utahensis* or *H. viridissima* using the primers that were used for *H. magnipapillata* and *H. oligactis*. This was not unexpected, because *H. utahensis* and *H. viridissima* are more distantly related to *H. vulgaris* than are *H. magnipapillata* and *H. oligactis*.<sup>2</sup>

***Sweet Tooth* Has Protein-tyrosine Kinase Activity**—To verify that *Sweet Tooth* is an active protein-tyrosine kinase, we expressed the kinase domain in the yeast *S. cerevisiae*. The absence of typical protein-tyrosine kinases in yeast (2) makes it a useful system for demonstrating protein-tyrosine kinase activity of heterologous proteins (12). Yeast containing a plasmid with the sequence encoding the *Sweet Tooth* kinase domain under control of the Gal4 promoter were grown in glucose and then transferred to galactose to induce expression of the *Sweet Tooth* protein. Protein-tyrosine kinase activity in the yeast cells was assayed by immunoblotting with an anti-phosphotyrosine antibody. An extract from the galactose-induced cells containing the *Sweet Tooth* kinase domain showed numerous phosphotyrosine-containing proteins (Fig. 4, lane 6), which were absent from cells grown in glucose (Fig. 4, lane 5). These results show that *Sweet Tooth* has the potential to be a ligand-activated protein-tyrosine kinase.

**Expression of the *Sweet Tooth* Gene**—To examine the expression pattern of the *Sweet Tooth* gene, whole-mount *in situ* hybridization with adult *Hydra* polyps was carried out using a probe mixture specific for the region of the cDNA encoding the extracellular domain. Hybridization of the probe mixture to poly(A)<sup>+</sup> RNA from adult *Hydra* polyps showed that it was specific for the *Sweet Tooth* gene, in that it detected a single RNA species of the expected size (Fig. 2).

The hybridization pattern we observed for whole animals is

<sup>2</sup> P. Snow and L. Buss, personal communication.

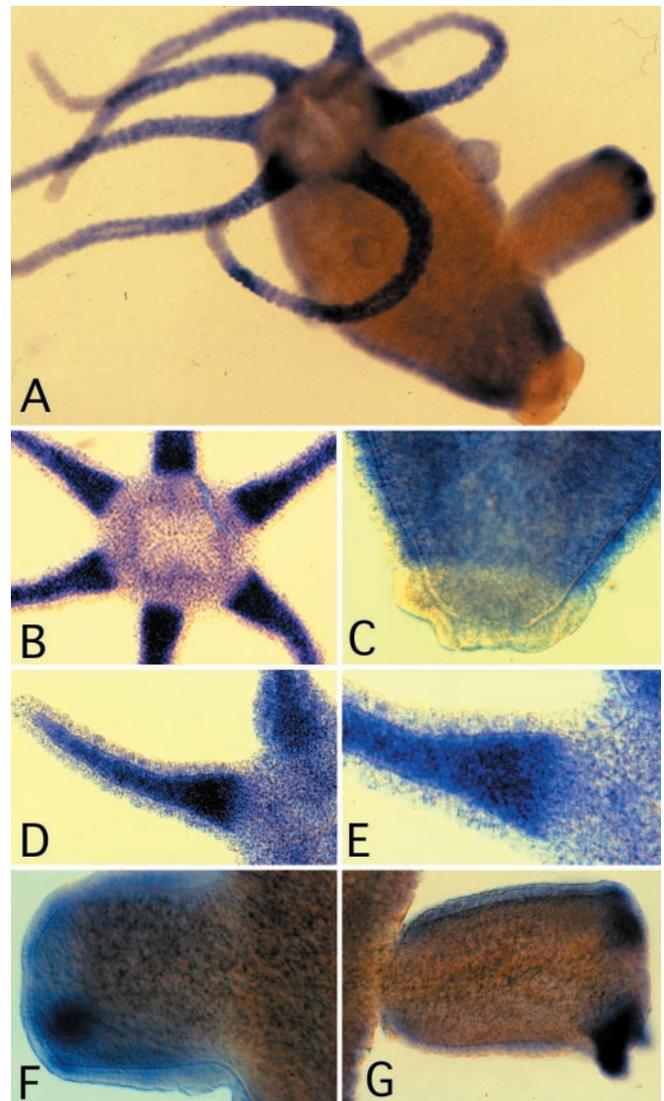


**FIG. 4. The kinase domain of *Sweet Tooth* is catalytically active.** The *Sweet Tooth* kinase domain was produced in yeast, and its activity was assayed by immunoblotting of total yeast protein with an anti-phosphotyrosine antibody. The *Src2* gene of *Xenopus laevis*, which has previously been shown to produce catalytically active protein in yeast (12), was used as a positive control. Uninduced cells were grown in glucose. Induced cells were grown in galactose. *Lane 1*, vector only, uninduced; *lane 2*, vector only, induced; *lane 3*, *Src2*, uninduced; *lane 4*, *Src2*, induced; *lane 5*, *Sweet Tooth*, uninduced; *lane 6*, *Sweet Tooth*, induced.

shown in Fig. 5A. The highest levels of *Sweet Tooth* message were observed in endodermal cells of the tentacles. The border of increased message level is located at the junction between cells of the tentacle and cells of the head. There is also a higher level of expression in the peduncle (the region between the foot basal disc and the budding zone) compared with the rest of the body column. A low level of expression is detected in the rest of the animal except in the foot basal disc cells, where expression is absent (Fig. 5C). The head of *Hydra* can be divided into two regions. The hypostome is the domed structure at whose apex the mouth opening is located. The tentacle zone is located at the base of the hypostome and consists of a ring of evenly spaced tentacles. To obtain a clearer view of the *Sweet Tooth* expression pattern in the head, we performed *in situ* hybridizations on decapitated heads. The result of such a hybridization (Fig. 5B) confirmed the result seen with intact polyps. The high level of RNA in the tentacle endoderm extends nearly the full length of the tentacle, fading only at the tentacle tip (Fig. 5D). From a higher-magnification view of the tentacle base it appears that the increase in *Sweet Tooth* RNA level in the tentacle endoderm occurs at least a few cells distal to the border between the tentacle and the body column (Fig. 5E).

By examining the distribution of *Sweet Tooth* RNA during the process of budding, *Hydra*'s mode of asexual reproduction, it was possible to determine when during tentacle formation the *Sweet Tooth* RNA level rises. The level of *Sweet Tooth* RNA begins to rise in the endoderm at the site of tentacle formation at or shortly before the time when evagination of the epithelial layers begins (Fig. 5F). Expression reaches its maximal level before the completion of tentacle growth (Fig. 5G). Tentacle development during the process of head regeneration was also examined with the same results (data not shown).

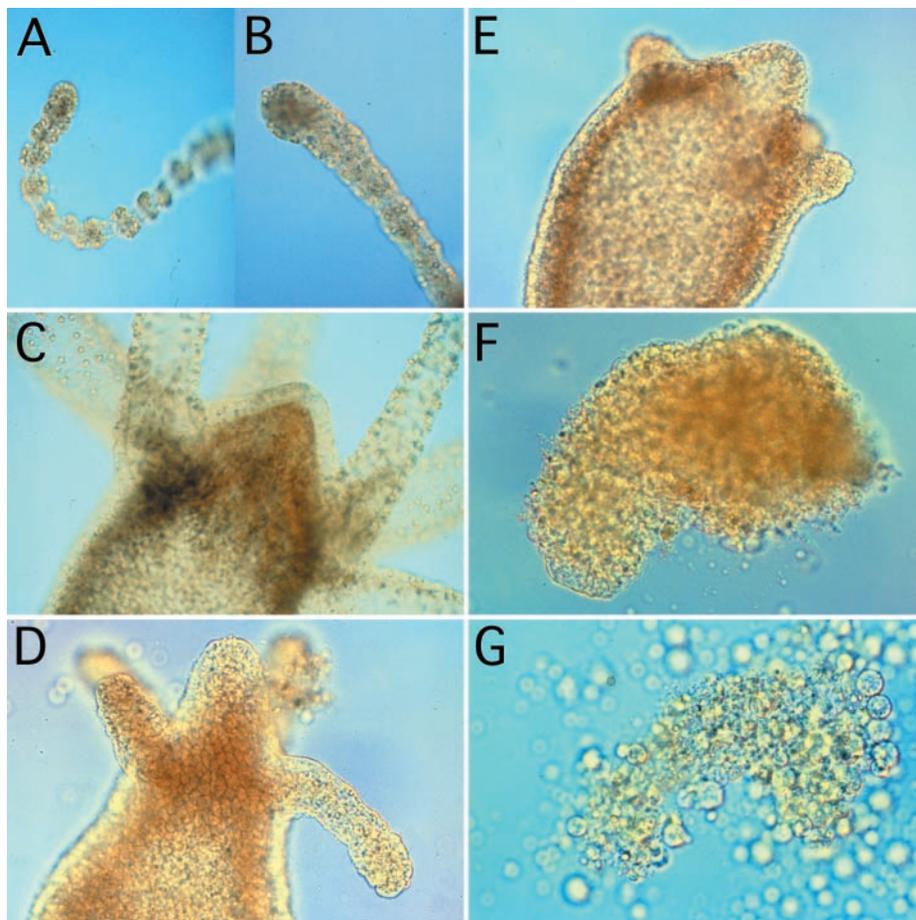
**Effects of Treatment of *Hydra* with Peptides Predicted to Block *Sweet Tooth* Ligand Binding**—To explore possible roles of *Sweet Tooth* in *Hydra*, we treated animals with synthetic peptides expected to block ligand binding. The peptides were designed based on previously published work examining the importance of amino acid residues 48–63 in the carbohydrate recognition domain (CRD) of selectins (25–27). Mutation of tyrosine 48 in the CRD of E-selectin to a phenylalanine abolishes carbohydrate binding (26). A synthetic peptide corresponding to the conserved residues 48–55 of E-, L-, and P-



**FIG. 5. Expression of *Sweet Tooth* in adult *Hydra* polyps detected by whole-mount *in situ* hybridization analysis.** A, distribution of *Sweet Tooth* RNA in the whole adult polyp. B, distribution of *Sweet Tooth* RNA in the hypostome and tentacles. Animals were decapitated before *in situ* hybridization. C, *Sweet Tooth* RNA distribution in basal portion of the adult polyp. D, view showing distribution of *Sweet Tooth* RNA in the tentacle. E, view of a tentacle focusing on the hypostome-tentacle border. F, *Sweet Tooth* RNA distribution in a stage 5 bud. G, *Sweet Tooth* RNA distribution in a bud between stages 6 and 7.

selectins (YYWIGIRK) was found to block the binding of all three selectins in both cell culture and animal systems (25). A synthetic peptide corresponding to residues 54–63 of any of the three selectins blocked binding of neutrophils to P-selectin (27).

Peptides containing the *Sweet Tooth* CTLD sequences corresponding to amino acids 48–53 of the E-selectin CRD (indicated in Fig. 1) were synthesized and purified by HPLC. In addition a scrambled version (LINGDYNW) of the CTLD1 peptide was produced. The peptide corresponding to CTLD2 was insoluble under the conditions we used and was eliminated from our studies. After ~10 days of treatment with peptide at a concentration of 100  $\mu$ M, animals began to lose cells rapidly, whereas controls in *Hydra* medium alone or treated with the scrambled peptide did not. Small piles of individual cells began to accumulate around the bases of the animals. After 10–11 days the tentacles began to shorten in length and swell at the tips (Fig. 6, A–D). At 12 days the heads of the treated animals had begun to bloat, the tentacles were reduced to small nubs (Fig. 6E), and many free cells were floating around the animals. At 13 days



**FIG. 6. Treatment of *Hydra* with peptides from a *Sweet Tooth* CTLD.** Representative polyps that were treated with the CTLD4 peptide (KYWIGLNK) or the control peptide (LLINGDYNW) at a concentration of 100  $\mu\text{M}$  are shown. *A*, apical portion of the tentacle of a polyp treated for 14 days with the control peptide. *B*, apical portion of the tentacle of a polyp treated for 14 days with the CTLD4 peptide. *C*, head region of a polyp treated for 14 days with the control peptide. *D*, head region of a polyp treated for 11 days with the CTLD4 peptide. *E*, head region of a polyp treated for 12 days with the CTLD4 peptide. *F*, polyp treated with the CTLD4 peptide for 13 days; the head end of the polyp (*right*) is disintegrating, whereas the foot basal disc (*lower left*) remains intact. *G*, remains of an animal after 14 days of treatment with the CTLD4 peptide.

the head had disintegrated, yet the foot remained largely intact (Fig. 6*F*). The animals disintegrated completely after  $\sim 14$  days (Fig. 6*G*). The CTLD4 peptide had the most rapid effect, with animals showing signs of cell loss and tentacle shrinkage 24–36 h before animals treated with the peptides for CTLD1 and CTLD3. If all three peptides were mixed, the time course of disintegration was the same as that for the CTLD4 peptide alone. Animals treated exactly as the experimental animals, but without peptides, and animals treated with the control peptide were unaffected. Raising the concentration of peptide to 1 mM only slightly accelerated the course of the response.

The presence of a high level of *Sweet Tooth* RNA in tentacle endodermal cells raised the possibility that *Sweet Tooth* may play a role in tentacle development. To test this possibility we attempted to block tentacle formation in regenerating animals and in budding animals. Animals were decapitated immediately below the tentacle zone and placed in peptide-containing medium. We also tested the animals' ability to regenerate after preincubation in peptide-containing medium for 5 days before decapitation. To analyze the effects of the reagents on budding, we treated animals containing stage 2–3 buds (28). It is during this stage in bud development that head structures begin to form. Exposure to peptides had no effect on regeneration or budding; however, animals did disintegrate in the manner described above after 14 days.

*Sweet Tooth* could act as an intercellular adhesion molecule. The fact that peptide-treated animals eventually disintegrate could be attributable to disruption of cell-cell adhesion. With *Hydra* there is a direct test for the ability of cells to adhere, that being the formation of aggregates of cells from dissociated animals. If *Hydra* polyps are dissociated into individual cells in a mild saline solution by gentle mechanical stimulation, the cells can be centrifuged into aggregates that reform the ecto-

dermal and endodermal epithelial layers, regenerate heads and feet, and eventually separate into complete animals (19). The complete morphogenesis of *Hydra* polyps from aggregates occurs in 4–7 days (19, 29). We added peptides before the final pelleting step in aggregate formation. After a 1-h incubation in peptide-containing medium, cells were pelleted and then transferred to fresh peptide-containing medium. We saw no changes in aggregate development or morphology in the presence of peptides. The animals did, however, disintegrate after 14 days.

#### DISCUSSION

As a result of the identification of RTK genes in the earliest diverging animal phyla, we now know that the ancestor of all modern metazoans contained signaling pathways activated by RTKs, but that such receptors are likely absent from both plants and unicellular animals. Studies with various model animal systems have revealed that RTKs play critical roles in developmental processes and in the regulation of cell division and physiology. The known RTK ligands are polypeptides, which are either released into the intercellular milieu or anchored to the surface of the cell. No RTKs have been identified that recognize carbohydrates, despite the diversity of carbohydrate molecules displayed on cell surfaces. Our identification of an RTK in *Hydra* that contains CTLDs in its extracellular region demonstrates that an RTK that potentially recognizes carbohydrates has appeared in at least one animal phylum. Cnidaria, the phylum of which *Hydra* is a member, diverged very early in the metazoan radiation. Thus RTKs containing CTLDs were potentially present in the last common ancestor of cnidarians and the rest of metazoans. If an RTK with CTLDs was present in this ancestor and conserved during subsequent stages of metazoan evolution, we would expect to find its progeny in most modern animals. So far this has not been the case.

Although C-type lectins have been identified in a number of metazoan phyla, none has been found to be an RTK. In *C. elegans*, the one case in which we have a complete inventory of RTKs for an animal, no gene encoding an RTK with CTLDs is present (21). However, *C. elegans* lacks several protein-tyrosine kinase genes that are present in *Hydra* and vertebrates (30), indicating that some genes have been lost in the lineage leading to *C. elegans*. Although *C. elegans* lacks RTKs with CTLDs, a data base search reveals that the nematode genome contains a number of genes encoding proteins with CTLDs. Before the cloning of the *Sweet Tooth* gene, no CTLD-containing protein had been identified in a diploblastic organism. Thus our data demonstrate that the CTLD arose before the divergence of triploblasts and diploblasts. Until more animal phyla are examined, we cannot say whether the linkage of CTLDs to an RTK occurred only in the phylum Cnidaria or whether it will turn out to be of more widespread occurrence.

A key piece of information necessary to understand the role of *Sweet Tooth* in *Hydra* is the nature of the ligand(s) that it recognizes. Although we currently lack this information, the extracellular portion of *Sweet Tooth* has the potential for a variety of interactions. Each CTLD is potentially capable of binding to a distinct ligand. In addition, the A and B repeats may also recognize ligands. Thus *Sweet Tooth* is potentially capable of recognizing a number of different molecules. At least two of the CTLDs (CTLDs 1 and 4) of *Sweet Tooth* have features that suggest that they could bind carbohydrates. However, the presence of a CTLD is not, in and of itself, sufficient to allow one to conclude that the CTLD will bind carbohydrate. For example, CTLD-containing proteins serve as antifreeze proteins in the circulatory systems of some fish (31, 32). One of the families of receptors on natural killer lymphocytes contains CTLDs, but it appears that at least some of these domains recognize protein ligands rather than carbohydrate ligands (33, 34). However, in the cases of CTLDs that do not bind carbohydrates, the CTLD sequences are less conserved than is the case for the *Sweet Tooth* CTLDs. For example, the CTLDs of natural killer cell receptors lack calcium binding site 2 (35). Although we have not yet identified ligands for *Sweet Tooth*, we have demonstrated that its kinase domain is catalytically active. Thus it is very likely that *Sweet Tooth* responds to ligand binding by activating one or more signal transduction pathways in the manner of other RTKs.

Given the possibility that *Sweet Tooth* is a receptor that is activated by binding to one or more carbohydrate ligands, what roles might it play in *Hydra*? Because of the simplicity of both the composition and organization of *Hydra*, the number of possible roles for *Sweet Tooth* is relatively limited. Two possibilities seem likely. First, *Sweet Tooth* may serve as an adhesion molecule, with adhesion being achieved by binding of the CTLDs to carbohydrates and/or proteins on adjacent cells. C-type lectins are known to act as adhesion molecules. The selectins mediate the binding of leukocytes to endothelial cells (36). Lecticans, proteoglycans that contain a C-type lectin domain and bind sulfated glycolipids (37), appear to play a role in cell adhesion in the nervous system (38). Our attempts to block adhesion of *Hydra* cells with synthetic peptides corresponding to sequences in the *Sweet Tooth* CTLDs were unsuccessful. This result suggests that *Sweet Tooth* may not be involved in adherence of *Hydra* cells to each other. Alternatively, adhesion could be mediated by several different molecules, with any one molecule being dispensable. An example of such redundancy among adhesion molecules is seen in *Drosophila*, in which loss of function of fasciclin I, a neural cell adhesion molecule, has no effect on development of the nervous system (39).

In addition to testing for a role in adhesion, we also tested

whether the *Sweet Tooth* peptides could block regeneration of the head, a process that requires adhesion as well as other morphogenetic processes. The high level of *Sweet Tooth* gene expression in the tentacles and during tentacle formation suggests the possibility that it might be involved in morphogenetic processes associated with tentacle formation. In *Hydra*, tissue displacement occurs continuously in the adult (40, 41). Cells from the body column are continuously moving into the tentacles. Thus the morphological changes associated with the conversion of body column tissue into tentacle tissue occur continuously. In addition to cell shape changes, this conversion almost certainly involves changes in cell adhesion. *Sweet Tooth* is thus a reasonable candidate for mediating such changes. To test this possibility, we carried out peptide treatment on animals that were regenerating heads. The peptides had no effect on the rate or course of regeneration. It has been demonstrated that head regeneration can be perturbed by treatment with both peptides and proteolytic fragments from extracellular matrix molecules (10, 42), indicating that application of peptides to regenerating animals is an appropriate method for testing whether a protein has a role in morphogenesis.

A third potential role for *Sweet Tooth* is in the recognition of foreign cells. *Hydra* has been shown to carry out xenorecognition. In heterografts of *H. oligactis* and *H. vulgaris* the cells of each species phagocytose the cells of the other species, with the ultimate outcome, over a period of several weeks, being the removal of all of the *H. vulgaris* cells from the grafted animal (43). C-type lectins have been shown to be used extensively for immune responses in both vertebrates and invertebrates (35, 44). Thus a lectin RTK is an attractive candidate for mediating xenorecognition in *Hydra*. We could envision, for example, that failure of *Sweet Tooth* to detect a species-specific carbohydrate on a cell with which it comes in contact could result in the registering of that cell as foreign and subsequent action against that cell. It is thus intriguing that treatment of *Hydra* with *Sweet Tooth* peptides results in the disintegration of the animal and that CTLD1 from *H. vulgaris* and *H. oligactis* shows sequence differences that suggest that they might recognize different carbohydrates.

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#### REFERENCES

- van der Geer, P., Hunter, T., and Lindberg, R. A. (1994) *Annu. Rev. Cell Biol.* **10**, 251–337
- Hunter, T., and Plowman, G. (1997) *Trends Biochem. Sci.* **22**, 18–22
- Steele, R. E., Lieu, P., Mai, N. H., Shenk, M. A., and Sarras, M. P., Jr. (1996) *Dev. Genes Evol.* **206**, 247–259
- Schacke, H., Rinkevich, B., Gamulin, V., Müller, I. M., and Müller, W. E. (1994) *J. Mol. Recognit.* **7**, 273–276
- Schacke, H., Schroder, H. C., Gamulin, V., Rinkevich, B., Müller, I. M., and Müller, W. E. (1994) *Mol. Membr. Biol.* **11**, 101–107
- Pulido, D., Campuzano, S., Koda, T., Modolell, J., and Barbacid, M. (1992) *EMBO J.* **11**, 391–404
- Lenhoff, H. M., and Brown, R. D. (1970) *Lab. Anim.* **4**, 139–154
- Chan, T. A., Chu, C. A., Rauen, K. A., Kroeher, M., Tatarewicz, S. M., and Steele, R. E. (1994) *Oncogene* **9**, 1253–1259
- Bosch, T. C. G., Unger, T. F., Fisher, D. A., and Steele, R. E. (1989) *Mol. Cell. Biol.* **9**, 4141–4151
- Sarras, M. P., Jr., Yan, L., Grens, A., Zhang, X., Agbas, A., Huff, J. K., St. John, P. L., and Abrahamson, D. R. (1994) *Dev. Biol.* **164**, 312–324
- Shenk, M. A., Bode, H. R., and Steele, R. E. (1993) *Development* **117**, 657–667
- Steele, R. E., Unger, T. F., Mardis, M. J., and Fero, J. B. (1989) *J. Biol. Chem.* **264**, 10649–10653
- Liu, H., Krizek, J., and Bretscher, A. (1992) *Genetics* **132**, 665–673
- Ito, H., Fukuda, Y., Murata, K., and Kimura, A. (1983) *J. Bacteriol.* **153**, 163–168
- Yaffe, M. P., and Schatz, G. (1984) *Proc. Natl. Acad. Sci. U. S. A.* **81**, 4819–4823
- Davis, R. W., Thomas, M., Cameron, J., John, T. P. S., Scherer, S., and Padgett, R. A. (1980) *Methods Enzymol.* **65**, 404–411
- Grens, A., Mason, E., Marsh, J. L., and Bode, H. R. (1995) *Development* **121**, 4027–4035
- Martinez, D. E., Dirksen, M.-L., Bode, P. M., Jamrich, M., Steele, R. E., and Bode, H. R. (1997) *Dev. Biol.* **523**–536

19. Gierer, A., Berking, S., Bode, H., David, C. N., Flick, K., Hansmann, G., Schaller, H., and Trenkner, E. (1972) *Nat. New Biol.* **239**, 98–101
20. Technau, U., and Holstein, T. W. (1995) *Development* **121**, 1273–1282
21. Ruvkun, G., and Hobert, O. (1998) *Science* **282**, 2033–2041
22. Weis, W. I., Kahn, R., Fourme, R., Drickamer, K., and Hendrickson, W. A. (1991) *Science* **254**, 1608–1615
23. Drickamer, K. (1992) *Nature* **360**, 183–186
24. Kolatkar, A. R., and Weis, W. I. (1996) *J. Biol. Chem.* **271**, 6679–6685
25. Briggs, J. B., Oda, Y., Gilbert, J. H., Schaefer, M. E., and Macher, B. A. (1995) *Glycobiology* **5**, 583–588
26. Erbe, D. V., Wolitzky, B. A., Presta, L. G., Norton, C. R., Ramos, R. J., Burns, D. K., Rumberger, J. M., Rao, B. N., Foxall, C., Brandley, B. K., and Lasky, L. A. (1992) *J. Cell Biol.* **119**, 215–227
27. Geng, J.-G., Heavner, G. A., and McEver, R. P. (1992) *J. Biol. Chem.* **267**, 19846–19853
28. Otto, J. J., and Campbell, R. D. (1977) *J. Exp. Zool.* **200**, 417–428
29. Bode, H. R. (1974) *Am. Zool.* **14**, 543–550
30. Steele, R. E., Stover, N. A., and Sakaguchi, M. (1999) *Gene (Amst.)* **239**, 91–97
31. Loewen, M. C., Gronwald, W., Sonnichsen, F. D., Sykes, B. D., and Davies, P. L. (1998) *Biochemistry* **37**, 17745–17753
32. Gronwald, W., Loewen, M. C., Lix, B., Daugulis, A. J., Sonnichsen, F. D., Davies, P. L., and Sykes, B. D. (1998) *Biochemistry* **37**, 4712–4721
33. Braud, V. M., Allan, D. S., O'Callaghan, C. A., Soderstrom, K., D'Andrea, A., Ogg, G. S., Lazetic, S., Young, N. T., Bell, J. I., Phillips, J. H., Lanier, L. L., and McMichael, A. J. (1998) *Nature* **391**, 795–799
34. Boyington, J. C., Riaz, A. N., Patamawenu, A., Coligan, J. E., Brooks, A. G., and Sun, P. D. (1999) *Immunity* **10**, 75–82
35. Weis, W. I., Taylor, M. E., and Drickamer, K. (1998) *Immunol. Rev.* **163**, 19–34
36. Bevilacqua, M. P., and Nelson, R. M. (1993) *J. Clin. Invest.* **91**, 379–387
37. Iozzo, R. V. (1998) *Annu. Rev. Biochem.* **67**, 609–652
38. Miura, R., Aspberg, A., Ethell, I. M., Hagihara, K., Schnaar, R. L., Ruoslahti, E., and Yamaguchi, Y. (1999) *J. Biol. Chem.* **274**, 11431–11438
39. Elkins, T., Zinn, K., McAllister, L., Hoffmann, F. M., and Goodman, C. S. (1990) *Cell* **60**, 565–575
40. Campbell, R. D. (1973) *J. Cell Sci.* **13**, 651–661
41. Campbell, R. D. (1967) *J. Morphol.* **121**, 19–28
42. Zhang, X., Hudson, B. G., and Sarras, M. P., Jr. (1994) *Dev. Biol.* **164**, 10–23
43. Bosch, T. C. G., and David, C. N. (1986) *J. Exp. Zool.* **238**, 225–234
44. Vasta, G. R., Ahmed, H., and Quesenberry, M. S. (1996) in *New Directions in Invertebrate Immunology* (Söderhäll, K., Iwanaga, S., and Vasta, G. R., eds) pp. 189–227, SOS Publications, Fair Haven, NJ
45. Drickamer, K., Dordal, M. S., and Reynolds, L. (1986) *J. Biol. Chem.* **261**, 6878–6887
46. Leung, J. O., Holland, E. C., and Drickamer, K. (1985) *J. Biol. Chem.* **260**, 12523–12527
47. Bevilacqua, M. P., Stengelin, S., Gimbrone, M. A., Jr., and Seed, B. (1989) *Science* **243**, 1160–1165
48. Altling-Mees, M. A., Sorge, J. A., and Short, J. M. (1992) *Methods Enzymol.* **216**, 483–495