Oncogene (2000) 19, 3925–3930
© 2000 Macmillan Publishers Ltd All rights reserved 0950–9232/00 $15.00
www.nature.com/onc

The Src/Csk regulatory circuit arose early in metazoan evolution

Michael A Miller[1,2], Imrana A Malik[1], Michael A Shenk[1,3] and Robert E Steele*,[1]

[1]*Department of Biological Chemistry and The Developmental Biology Center, University of California, Irvine, California, CA 92697, USA*

We have identified a gene encoding a member of the Csk family of non-receptor protein-tyrosine kinases (PTKs) in the early-diverging metazoan *Hydra*. *In situ* hybridization analysis of the distribution of RNA from the *Hydra* Csk gene indicates that it is expressed in most of the epithelial cells of the adult polyp and in gametogenic cells. Comparison of the expression pattern of *Hydra* Csk with that of STK, the *Hydra* Src gene orthologue, reveals that the two genes are largely co-expressed. Such co-expression is consistent with a role for *Hydra* Csk in regulation of STK activity. This possibility was tested directly by co-expressing *Hydra* Csk with STK in yeast. Co-expression suppressed the growth inhibition seen when STK alone is expressed in yeast. Suppression was dependent on the presence of the putative regulatory tyrosine in the carboxyl-terminal tail of STK. Phosphotyrosine immunoblot analysis confirmed that expression of Csk resulted in suppression of STK kinase activity. Taken together these data indicate that the regulatory circuit involving Src and Csk PTKs was established prior to the divergence of the phylum Cnidaria from the rest of the metazoans. *Oncogene* (2000) **19,** 3925–3930.

Keywords: evolution; metazoan; Src; Csk; protein-tyrosine kinase; Cnidaria

Introduction

Src family PTKs have been identified in the earliest diverging metazoan phyla (Bosch *et al*., 1989; Ottilie *et al*., 1992), suggesting that they play a critical role in the function of animal cells. Although the evolutionary history of Src PTKs is now relatively well known, we still know very little about the evolution of the pathways in which Src PTKs participate. An understanding of the evolution of these pathways will aid in the elucidation of the fundamental features of Src PTK function in animals.

Studies in vertebrates have demonstrated that the various members of the Src PTK family are negatively regulated through phosphorylation of their carboxyl-terminal tails by either Csk or Ctk, the two members of the Csk PTK family (Neet and Hunter, 1996). The importance of this regulatory mechanism has been demonstrated by disruption of the Csk gene in mice. Src PTKs in mice in which the Csk gene is inactivated have elevated kinase activity, and the animals die as embryos (Imamoto and Soriano, 1993; Nada *et al*., 1993). Comparison of the amino acid sequences of Src PTKs of invertebrates reveals the presence of a putative carboxyl-terminal regulatory tyrosine in every case. This finding suggested that a Csk kinase would be present in invertebrates and that it would regulate Src kinase activity by phosphorylation of the putative regulatory tyrosine. Confirmation of this hypothesis would indicate that the Src/Csk regulatory circuit is a fundamental feature of animal cells. Using PCR with primers encoding conserved amino acid sequences in the catalytic domain of PTKs we identified a Csk gene from the early-diverging animal *Hydra*. Furthermore, we have found that *Hydra* Csk regulates Src activity as it does in vertebrates.

Results

A gene encoding a member of the Csk family of protein-tyrosine kinases is present in Hydra

Using PCR in combination with primers encoding conserved amino acid sequences in the catalytic domain of PTKs, we have identified genes from the cnidarian *Hydra vulgaris* which encode non-receptor and receptor PTKs (Chan *et al*., 1994; Steele *et al*., 1996). One of the amplified fragments encoded a predicted amino acid sequence which was most closely related to the two vertebrate members of the Csk family. By a combination of cDNA library screening and RACE, we obtained the entire coding sequence of this gene. Comparison of the complete amino acid sequence for the predicted protein supported its initial assignment to the Csk family. An alignment of the *Hydra* Csk amino acid sequence with the human Csk sequence is shown in Figure 1. Like the vertebrate members of the Csk family, *Hydra* Csk contains SH3 and SH2 domains, lacks a carboxyl-terminal regulatory tyrosine and a myristoylation signal, and lacks activating tyrosine residues in the kinase domain (Nada *et al*., 1991; Neet and Hunter, 1996). Sequence conservation between *Hydra* Csk and the vertebrate Csk family members is high in the SH3 and SH2 domains and in the kinase domain.

Phylogenetic analysis of *Hydra* Csk confirms that it is a member of the Csk family and provides insight into the evolutionary history of the family. A maximum parsimony analysis was carried out using a combination of the sequences of the SH3, SH2, and kinase domains of representatives of the non-receptor

*Correspondence: RE Steele, Department of Biological Chemistry, 240D Medical Sciences I, University of California, Irvine, CA 92697-1700, USA
Current addresses: [2]Department of Cell Biology, Vanderbilt University School of Medicine, Nashville, TN 37232-2175, USA; [3]Genesis Research and Development Corporation Limited, P.O. Box 50, Auckland, New Zealand
Received 6 March 2000; revised 22 May 2000; accepted 31 May 2000

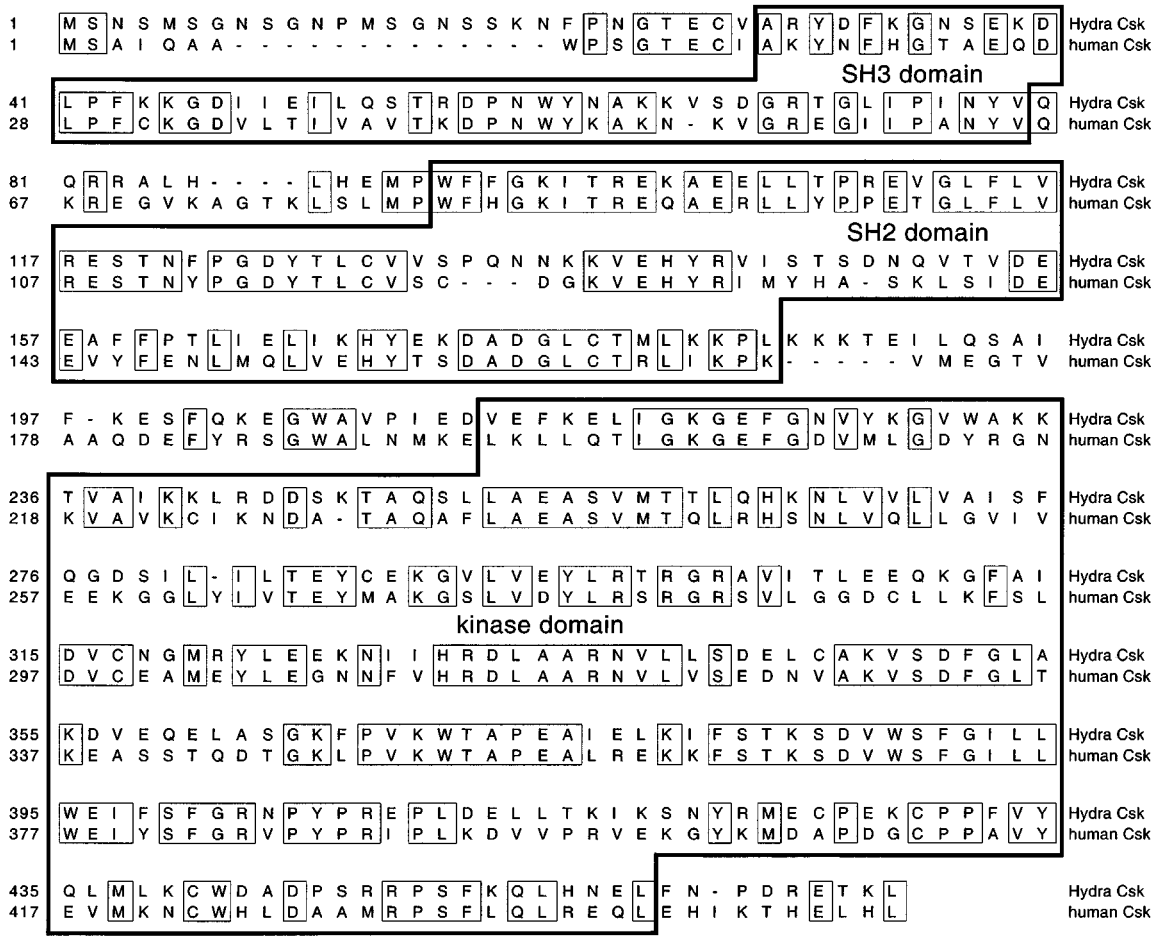

Figure 1 Alignment of the *Hydra* Csk amino sequence with the human Csk sequence. The sequences were aligned using CLUSTAL as implemented by the Megalign program of the LASERGENE™ package (DNASTAR). Amino acids which are identical in the two sequences are boxed. The boundaries of the SH3, SH2, and kinase domains are indicated by the boxes with thick lines. The positions of the ends of the SH3 and SH2 domains are from the GenBank entry for Human Csk. The positions of the ends of the kinase domain are from Hanks and Quinn (1991). The *Hydra* Csk sequence has been deposited in the GenBank database under Accession Number AF067775

PTK families which contain these three domains and also have a conserved intron location in the sequence encoding the SH2 domain. The results of this analysis (Figure 2) support the conclusion that a single Csk family member was present prior to the divergence of Cnidaria from the rest of the metazoans.

Phylogenetic analysis was also carried out using each of the three domains (SH3, SH2, and kinase domains) individually (data not shown). In all three cases the grouping of *Hydra* Csk with the vertebrate Csk family members was strongly supported (bootstrap values of 89–91). Thus *Hydra* Csk and the vertebrate Csk family members clearly arose from a common ancestor in which all three domains had been assembled.

The Hydra *Csk gene and the* Hydra *Src gene orthologue STK are co-expressed in cells of the adult polyp*

If Csk negatively regulates the kinase activity of STK in *Hydra*, then both genes should be expressed in the same cell types. Whole mount *in situ* hybridizations were performed on adult polyps using probes for STK and Csk. The expression patterns were broad and overlapping in both sexual and nonsexual animals (Figure 3). During oogenesis and spermatogenesis, expression of both genes was observed in both somatic cells and in the gametogeneic cells (Figure 3c–f). These results are consistent with the hypothesis that Csk regulates STK.

Hydra *Csk suppresses STK-induced growth inhibition in yeast*

Various studies have demonstrated that expression of vertebrate Src in the yeast *Saccharomyces cerevisiae* inhibits growth (Brugge *et al*., 1987; Kornbluth *et al*., 1987) and that co-expression of vertebrate Csk suppresses this inhibition (Murphy *et al*., 1993). Expression of STK in yeast also inhibits growth (Figure 4), suggesting that it phosphorylates one or more of the same yeast proteins as vertebrate Src. If *Hydra* Csk regulates STK activity, co-expression should suppress the growth inhibition. *Hydra* Csk showed the predicted suppression (Figure 4). Furthermore, suppression was not seen when a version of STK (STK-Y>F) lacking the predicted Csk phosphorylation site was used (Figure 4). Taken together these data support the hypothesis that *Hydra* Csk regulates STK activity by phosphorylating the carboxyl-terminal tyrosine which is conserved among all of the Src proteins characterized to date.

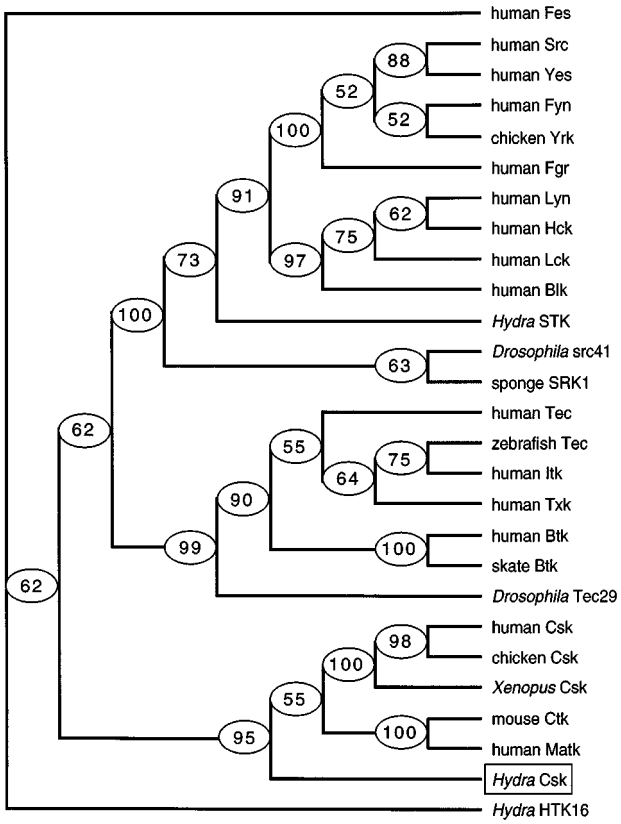

Figure 2 Results of a phylogenetic analysis of *Hydra* Csk and other metazoan non-receptor PTKs. The tree includes representatives of the three non-receptor PTK families which contain an SH3 and an SH2 domain and a conserved intron in the sequence encoding the SH2 domain. The analysis was performed with a combination of the sequences of the SH3, SH2, and kinase domains. The kinase domain sequences from the human Fes and *Hydra* HTK16 PTKs were used as outgroups. The single most parsimonious tree is shown. Circled numbers are bootstrap values. Sequences were obtained from the National Center for Biotechnology Information using the WWW Entrez Browser. The Entrez Sequence ID numbers for the sequences included in the analysis are as follows: human Src, 125711; human Yes, 125870; human Fyn, 4503823; chicken Yrk, 462471; human Fgr, 66801; human Lyn, 125480; human Hck, 4504357; human Lck, 125474; human Blk, 4502413; human Tec, 1174630; zebrafish (*Danio rerio*) Tec, 2353318; human Itk, 585361; human Btk, 4557377; skate (*Raja eglanteria*) Btk, 2228716; human Txk, 4507743; chicken Csk, 729886; *Xenopus laevis* Csk, 2967840; mouse Ctk, 2117810; human Matk, 4505109; *Hydra* STK, 159274; *Drosophila* src41, 1536790; sponge (*Spongilla lacustris*) SRK1, 1174436; human Tec, 1174630; *Drosophila* Tec29, 2827464; human Csk, 729887; *Hydra vulgaris* Csk, 3560565; human Fes, 400127; *Hydra vulgaris* HTK16, 392932. The kinase domain sequences were aligned using the kinase domain alignment database of Hanks and Quinn (1991) as a template. The SH2 and SH3 domain sequences were aligned using CLUSTAL X (Thompson *et al*., 1997)

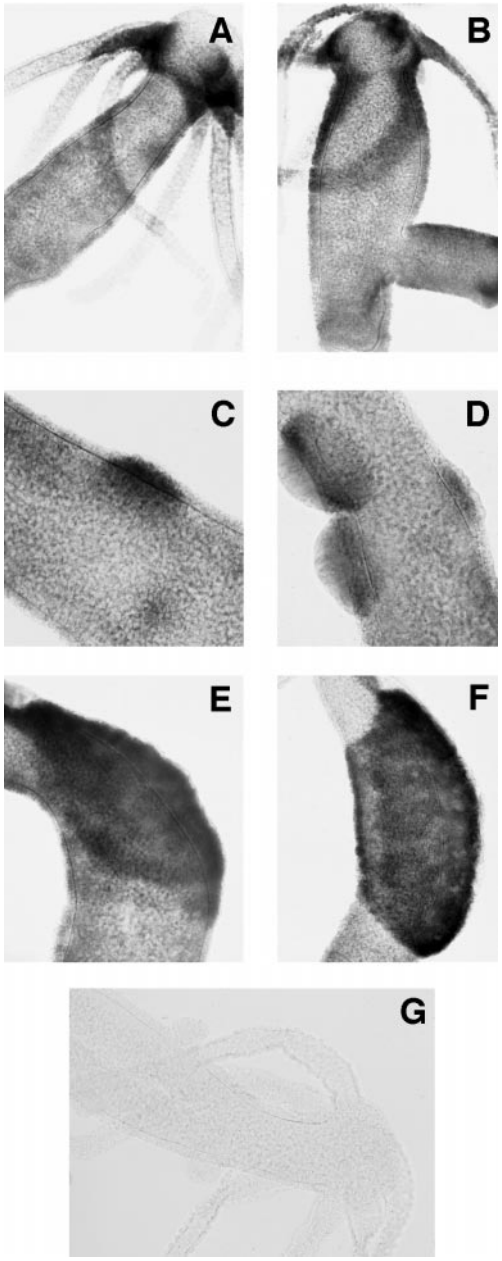

Figure 3 Expression of Csk and STK in adult *Hydra vulgaris* polyps. Fixed polyps were analysed by *in situ* hybridization with antisense RNA probes containing the coding sequence for Csk or STK. (**a**) Csk, asexual polyp; (**b**) STK, asexual polyp; (**c**) Csk, sexual male polyp; (**d**), STK, sexual male polyp; (**e**) Csk, sexual female polyp; (**f**) STK, sexual female polyp; (**g**) control in which a Csk sense probe was used. Sexual male polyps contain one (**c**) or more (**d**) testes. Sexual female polyps contain the large ectodermal

A decrease in protein phosphorylation on tyrosine correlates with relief of STK-induced yeast growth suppression by Hydra *Csk*

To confirm that the relief of STK-induced growth suppression in yeast was due to a change in tyrosine phosphorylation, we examined the activities of the *Hydra* kinases by immunoblot analysis with an antiphosphotyrosine antibody. *S. cerevisiae* lacks canonical protein-tyrosine kinases (Hunter and Plowman, 1997) and thus provides a useful system for examining the activities of such enzymes. Yeast producing either wild type STK or the STK Y>F mutant contained a large number of proteins phosphorylated on tyrosine (Figure 5, lanes 6–7). These phosphorylations were absent in yeast containing only the vector (Figure 5, lane 1) or yeast in which synthesis of STK was not induced (Figure 5, lane 5). Co-expression of STK and Csk resulted in a marked reduction in the number of proteins phosphorylated on tyrosine (Figure 5, lane 8). Furthermore, the predominant protein in this case had a molecular weight of 57 kDa, the predicted size of the STK protein (Bosch *et al*., 1989). As expected, Csk had no effect on tyrosine phosphorylation in cells expressing the Y>F mutant of STK (Figure 5, lane 7). These results are consistent with the growth data in Figure 4 and support the conclusion

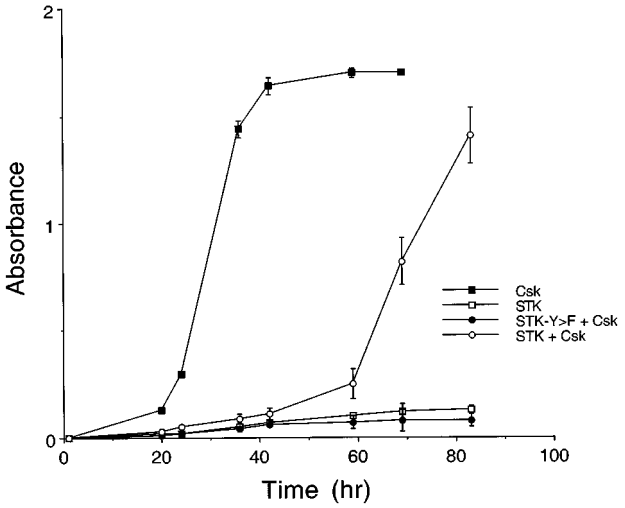

Figure 4 Effect of *Hydra* Csk on STK-induced inhibition of yeast growth. The growth of *S. cerevisiae* cells expressing STK, STK-Y>F, or Csk, alone or in combination, were monitored by measuring the absorbance of the cultures at 600 nm. Cultures were initiated in the presence of glucose and expression of the *Hydra* genes was induced by replacement of glucose with galactose. The growth of three to five cultures was followed for each of the strains. Error bars represent the standard deviations of the means. STK-Y>F is a mutant version of STK in which the putative carboxyl-terminal regulatory tyrosine has been changed to phenylalanine

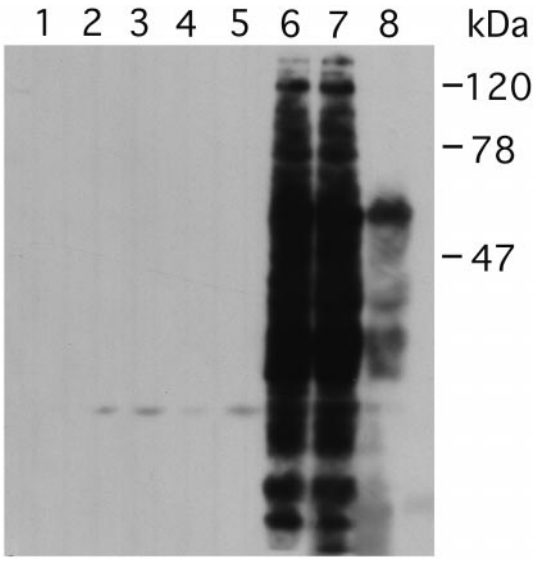

Figure 5 Effect of *Hydra* Csk on tyrosine phosphorylation in yeast expressing STK. Equal amounts of protein from yeast cells grown as in Figure 4 were fractionated by SDS–PAGE and probed with an anti-phosphotyrosine antibody. Lane 1, strain containing the URA3 and LEU2 vectors (see Materials and methods) and grown in glucose. Lane 2, identical to lane 1 except grown in galactose. Lane 3, strain containing Csk (in the LEU2 vector) plus empty URA3 vector and grown in glucose. Lane 4, identical to lane 3 except grown in galactose. Lane 5, strain containing STK (in the URA3 vector) plus empty LEU2 vector and grown in glucose. Lane 6, identical to lane 5 except grown in galactose. Lane 7, strain containing STK Y>F (in the URA3 vector) plus Csk (in the LEU2 vector) and grown in galactose. Lane 8, strain containing STK (in the URA3 vector) plus Csk (in the LEU2 vector) and grown in galactose. The positions of the pre-stained high range molecular size markers (Bio-Rad) are indicated

that phosphorylation of STK on its predicted regulatory tyrosine leads to suppression of kinase activity.

Discussion

Src genes have now been identified in a number of invertebrate phyla including Porifera (sponges) (Ottilie *et al*., 1992). Isolation of Src genes from a sponge proved that a Src gene was present in the last common ancestor of all modern metazoa. Further, the regulatory tyrosine residue that Csk phosphorylates in vertebrates is conserved in invertebrate Src proteins, suggesting the possibility that regulation of Src activity by Csk appeared early in metazoan evolution. Our data demonstrate that this regulatory interaction was established prior to the divergence of Cnidaria from the rest of the metazoans. The Src/Csk circuit is thus a fundamental feature of animal cell signal transduction. Recently, a fragment of what is likely an orthologue of the Csk gene was isolated from a sponge (Suga *et al*., 1999). This result argues that both Csk and Src were present in the last common ancestor of all modern metazoans. It will be of interest to determine whether sponge Csk also regulates Src activity. The recent identification of a Csk gene in *C. elegans* (Plowman *et al*., 1999) will allow the application of genetic methods to further dissect the evolution of the Src/Csk regulatory interaction.

Of obvious interest is the question of how the Src/Csk regulatory circuit evolved. The conservation of protein domain organization between Src and Csk and intron locations in their genes (Brauninger *et al*., 1993) indicates that these two genes arose as the result of the duplication of a gene encoding a non-receptor PTK with SH3 and SH2 domains. How the regulatory interaction then evolved is not clear. It is, however, interesting that Src itself apparently has some capacity to phosphorylate its regulatory tyrosine (Cooper and Runge, 1987). Gene duplication followed by improvement of this inherent capacity in the new gene product (ancestral Csk) may have been part of the process of Csk evolution.

Materials and methods

Hydra *culture*

A clone of the Zurich strain of *Hydra vulgaris* was obtained from Dr Monika Hassel (Heidelberg) and maintained according to standard methods (Lenhoff and Brown, 1970).

Polymerase chain reaction (PCR)

Amplification and cloning of cDNA fragments from genes encoding PTKs was carried out as previously described (Chan *et al*., 1994; Steele *et al*., 1996). Clones were screened by sequencing using only the dideoxy-T stop reaction mixture (T-tracking). The complete sequence of an example of each clone type was determined using the T7 Sequenase™ kit from Amersham Life Science.

Library screening and DNA sequencing

A clone containing all but the 3′ end of the coding region of the *Hydra* Csk gene was isolated by screening of a cDNA library as previously described (Steele *et al*., 1999). This clone

was sequenced by primer walking using the services of the UCI DNA Core Facility.

Rapid amplification of cDNA ends (RACE)

A cDNA fragment containing the 3′ end of the coding region of *Hydra* Csk was isolated using the RACE technique (Frohman *et al*., 1988). An aliquot of a *Hydra vulgaris* cDNA library (Sarras *et al*., 1994) was used as the template. The primers that were used corresponded to the 3′ Csk sequence and the phage vector sequences adjacent to the site into which the cDNA inserts were cloned.

Phylogenetic analysis

A phylogenetic analysis was performed using a maximum parsimony method. The analysis was carried out using the combined kinase, SH3, and SH2 domain sequences of representatives of the non-receptor PTK families which contain these three domains and also have a conserved intron location in the sequence encoding the SH2 domain. The human Fes and *Hydra* HTK16 kinase domain sequences were used as outgroups. The heuristic search option of PAUP 3.1.1 (Swofford, 1993) was used, with 500 random sequence taxon addition replicates and tree bisection and reconnection branch swapping. The 'protpars' matrix of PAUP 3.1.1 was used to weight amino acid substitutions. To obtain bootstrap values, 100 bootstrap replicates were done using simple taxon addition with tree bisection and reconnection branch swapping.

Protein expression in yeast

A fragment containing the coding sequence of *Hydra* STK was obtained by amplification with PfuTurbo DNA polymerase (Stratagene) from a cDNA clone using gene-specific primers that included a *Bam*HI cleavage site at the 5′ end of the 5′ primer and a *Sal*I cleavage site at the 5′ end of the 3′ primer. The 5′ primer also included the eight nucleotides upstream of the ATG in the GAL4 gene (5′-CCTGAAAG-3′). The Csk coding sequence was amplified in a similar manner to that used for STK except that the 3′ primer included the sequence encoding the carboxyl terminal tail identified by RACE (see above). 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 URA3 galactose-inducible yeast expression vector pRS316-GAL1 (Liu *et al*., 1992). To prepare LEU2 galactose-inducible constructs, inserts were transferred from pRS316-GAL1 into the LEU2 vector pRS315 (Sikorski and Hieter, 1989) containing the GAL1 promoter from pRS316-GAL1. Strain W303 of *Saccharomyces cerevisiae* was transformed with plasmid DNAs using the lithium acetate method (Ito *et al*., 1983). All transformations were done with either LEU2 and URA3 vectors each containing a *Hydra* gene (e.g. STK and Csk) or with one vector containing a *Hydra* gene (e.g. Csk) and the other vector without an insert. Transformants were selected on minimal medium lacking uracil and leucine. For expression of the *Hydra* proteins, plasmid-containing yeast cells were grown at 30°C in uracil-minus leu-minus minimal medium plus glucose overnight and then diluted 1 : 500 in uracil-minus leu-minus minimal medium plus galactose and grown for the times indicated. Growth was measured by absorbance at 600 nm. The STK Y>F mutant cDNA was created by standard PCR mutagenesis methods (Higuchi *et al*., 1988; Vallette *et al*., 1989).

Immunoblot analysis of tyrosine phosphorylation

Yeast cells were harvested by centrifugation and proteins were extracted by alkaline lysis (Yaffe and Schatz, 1984). Protein concentrations were determined using the BCA Protein Assay Reagent Kit (Pierce). Equal amounts of protein were fractionated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to an Immobilon-P filter (Millipore) using a Bio-Rad Trans-Blot SD Semi-Dry 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) which had been diluted 1 : 13 000 in Tris-buffered saline plus 0.05% Tween 20 (TBST). The filter was subsequently washed at room temperature five times for 5 min each in TBST. After incubation for 1 h at room temperature with a horseradish peroxidase-conjugated goat anti-mouse IgG antibody (Transduction Laboratories; diluted 1 : 25 000 in TBST), the filter was washed at room temperature four times for 5 min each in TBST. Bound antibody was detected with the SuperSignal chemiluminescent substrate for Western blotting (Pierce).

In situ *hybridization*

Preparation of digoxygenin-labeled RNA probes and whole-mount *in situ* hybridization to *Hydra vulgaris* polyps were carried out essentially as previously described (Grens *et al*., 1995; Martínez *et al*., 1997). Polyps were mounted in Euparal™ (Asco Laboratories) and photographed using the bright field optics of an Olympus Vanox microscope and Ektachrome™ 160T film (Kodak).

Acknowledgments
We thank Diane Bridge for her critical reading of the manuscript and for numerous and always illuminating discussions of evolutionary biology. Support for this work was provided by National Science Foundation grant IBN-9808828 to RE Steele. IA Malik was supported by the UCI Summer Undergraduate Research Fellowship (SURF) Program. MA Shenk was supported by an NIH training grant to the UCI Developmental Biology Center.

References

Bosch TCG, Unger TF, Fisher DA and Steele RE. (1989). *Mol. Cell. Biol.,* **9,** 4141 – 4151.
Brauninger A, Karn T, Strebhardt K and Rubsamen-Waigmann H. (1993). *Oncogene,* **8,** 1365 – 1369.
Brugge JS, Jarosik G, Andersen J, Queral-Lustig A, Fedor-Chaiken M and Broach JR. (1987). *Mol. Cell. Biol.,* **7,** 2180 – 2187.
Chan TA, Chu CA, Rauen KA, Kroiher M, Tatarewicz SM and Steele RE. (1994). *Oncogene,* **9,** 1253 – 1259.
Cooper JA and Runge K. (1987). *Oncogene Res.,* **1,** 297 – 310.
Frohman MA, Dush MK and Martin GR. (1988). *Proc. Natl. Acad. Sci. USA,* **85,** 8998 – 9002.
Grens A, Mason E, Marsh JL and Bode HR. (1995). *Development,* **121,** 4027 – 4035.
Hanks SK and Quinn AM. (1991). *Meth. Enzymol.,* **200,** 38 – 62.
Higuchi R, Krummel B and Saiki RK. (1988). *Nucl. Acids Res.,* **16,** 7351 – 7367.
Hunter T and Plowman G. (1997). *Trends Biochem. Sci.,* **22,** 18 – 22.

Imamoto A and Soriano P. (1993). *Cell,* **73,** 1117–1124.
Ito H, Fukuda Y, Murata K and Kimura A. (1983). *J. Bacteriol.,* **153,** 163–168.
Kornbluth S, Jove R and Hanafusa H. (1987). *Proc. Natl. Acad. Sci. USA,* **84,** 4455–4459.
Lenhoff HM and Brown RD. (1970). *Lab. Anim.,* **4,** 139–154.
Liu H, Krizek J and Bretscher A. (1992). *Genetics,* **132,** 665–673.
Martínez DE, Dirksen M-L, Bode PM, Jamrich M, Steele RE and Bode HR. (1997). *Dev. Biol.,* **192,** 523–536.
Murphy SM, Bergman M and Morgan DO. (1993). *Mol. Cell. Biol.,* **13,** 5290–5300.
Nada S, Okada M, MacAuley A, Cooper JA and Nakagawa H. (1991). *Nature,* **351,** 69–72.
Nada S, Yagi T, Takeda H, Tokunaga T, Nakagawa H, Ikawa Y, Okada M and Aizawa S. (1993). *Cell,* **73,** 1125–1135.
Neet K and Hunter T. (1996). *Genes Cells,* **1,** 147–169.
Ottilie S, Raulf F, Barnekow A, Hannig G and Schartl M. (1992). *Oncogene,* **7,** 1625–1630.
Plowman GD, Sudarsanam S, Bingham J, Whyte D and Hunter T. (1999). *Proc. Natl. Acad. Sci. USA,* **96,** 13603–13610.
Sarras Jr MP, Yan L, Grens A, Zhang X, Agbas A, Huff JK, St John PL and Abrahamson DR. (1994). *Dev. Biol.,* **164,** 312–324.
Sikorski RS and Hieter P. (1989). *Genetics,* **122,** 19–27.
Steele RE, Lieu P, Mai NH, Shenk MA and Sarras Jr MP. (1996). *Dev. Genes Evol.,* **206,** 247–259.
Steele RE, Stover NA and Sakaguchi M. (1999). *Gene,* **239,** 91–97.
Suga H, Koyanagi M, Hoshiyama D, Ono K, Iwabe N, Kuma K and Miyata T. (1999). *J. Mol. Evol.,* **48,** 646–653.
Swofford DL. (1993). PAUP: Phylogenetic analysis using parsimony, version 3.1.1. Illinois Natural History Survey, Champaign: Illinois.
Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F and Higgins DG. (1997). *Nucl. Acids Res.,* **25,** 4876–4882.
Vallette F, Mege E, Reiss A and Adesnik M. (1989). *Nucl. Acids Res.,* **17,** 723–733.
Yaffe MP and Schatz G. (1984). *Proc. Natl. Acad. Sci. USA,* **81,** 4819–4823.