Preferential expression of a pp60ᶜ⁻ˢʳᶜ related protein tyrosine kinase activity in nerve cells of the early metazoan Hydra (Coelenterates)

Manfred Schartl[1], Thomas Holstein[2], Scott M. Robertson[1] & Angelika Barnekow[3]

[1]Gene Center, Max-Planck-Institute for Biochemistry, Am Klopferspitz 18a, D-8033 Martinsried; [2]Zoological Institute, Luisenstrasse 14, D-8000 München 2; [3]Institute for Medical Virology, Frankfurterstrasse 107, D-6300 Giessen, Federal Republic of Germany

It has been suggested that the proto-oncogene c-src plays a functional role in developing neurons, and in the mature nerve cells of higher vertebrates. The coelenterate Hydra represents the most primitive known organism possessing nerve cells. With Southern blot hybridizations we have demonstrated src-related sequences in Hydra. Antisera specific for the c-src gene product (pp60ᶜ⁻ˢʳᶜ) of birds and mammals precipitate a protein from Hydra cell extracts with a tyrosine-specific protein kinase activity. Studies of tissues and cells fractionated from a temperature sensitive mutant of Hydra which is depleted of interstitial (including nerve) cells at the non-permissive temperature, have indicated the src-like kinase of Hydra to be preferentially expressed in nerve cells. The high conservation of structural features and of the expression pattern indicates a basic function for pp60ᶜ⁻ˢʳᶜ in neurons.

Introduction

Proto-oncogenes, the cellular homologs of retroviral transforming genes, have been implicated in the regulation of normal cellular processes such as proliferation and differentiation. The c-src gene was the first cellular proto-oncogene identified (Stehelin et al., 1976). It encodes a phosphoprotein of 60 000 daltons (pp60ᶜ⁻ˢʳᶜ). This cellular protein, like its viral counterpart, possesses a tyrosine-specific protein kinase activity (for review see Hunter & Cooper, 1985). However, despite this salient feature the normal physiological role of the c-src gene product remains unknown.

Investigations on the phylogenetic distribution of the c-src gene and its evolutionary precedents, respectively, have indicated that this gene appears first during phylogenesis in the most primitive multicellular animals, the sponges (Barnekow & Schartl, 1984), and might be as old as 1.5 billion years. The sequence of the c-src gene has been found to be highly conserved in human (Anderson et al., 1985), mouse (Martinez et al., 1987), chicken (Takeya & Hanafusa, 1983), fish (S.M. Robertson et al., submitted) and Drosophila (Hoffmann-Falk et al., 1983; Simon et al., 1985). In addition, the enzymatic properties of pp60ᶜ⁻ˢʳᶜ have been shown to be comparable in all metazoans tested so far (Schartl & Barnekow, 1982; Barnekow & Schartl, 1987). This evolutionary conservation points to a crucial function for this gene in multicellular animals. The fact that this gene under certain conditions has a transforming capability also suggests that it plays an integral role in the

regulation of normal cellular homeostasis. Expression studies on the pp60ᶜ⁻ˢʳᶜ protein and c-src mRNA have revealed that cells of the neural cell lineage preferentially express the c-src gene, although it has been found that differentiating monomyelocytic cells in vitro (Barnekow & Gessler, 1986; Gee et al., 1986) and blood platelets (Golden et al., 1986) also express the gene at elevated levels. Neural organs contain both high amounts of pp60ᶜ⁻ˢʳᶜ and its kinase activity (Cotton & Brugge, 1983; Schartl & Barnekow, 1984; Fults et al., 1985; Maness, 1986) and c-src mRNA (Gonda et al., 1982; Simon et al., 1985; Gessler & Barnekow, 1984; Vardimon et al., 1986; Mäueler et al., 1988). During differentiation of neural cells of higher vertebrates an altered form of pp60ᶜ⁻ˢʳᶜ is expressed (Brugge et al., 1985; Lynch et al., 1986; Le Beau et al., 1987; Cartwright et al., 1987; Brugge et al., 1987), which is encoded by a unique mRNA that contains in contrast to the corresponding molecule from non-neural cells a six amino-acid insertion, apparently due to a differential splicing mechanism (Martinez et al., 1987; Levy et al., 1987; Raulf et al., 1989).

Based on the striking nerve cell-specific expression of c-src and in particular studies on c-src expression during development (Schartl & Barnekow, 1984; Lynch et al., 1986; Cartwright et al., 1987; Mäueler et al., 1988; Fults et al., 1985; Gessler & Barnekow, 1984; Vardimon et al., 1986; Levy et al., 1984; Sorge et al., 1984) it was deduced that the c-src gene product primarily plays a role during cytodifferentiation of nerve cells. The coelenterate Hydra offers a unique experimental system in which to investigate in more detail the role of the c-src gene in differentiation of the nerve cell lineage. Coelenterates are the most primitive animals which have a developed nervous system. Differentiation of nerve cells in Hydra occurs continuously from a population of pluripotent stem cells (interstitial cells; I cells), which are uniformly distributed along the body column (gastric region) and which can also give rise to nematoblasts, nematocytes, or as a minor fraction to gland cells (for review see Bode & David, 1978; David et al., 1986). The pattern of nerve and nematocyte differentiation however is not uniform throughout the body column: nerve differentiation occurs primarily in head and foot tissue, whereas nematocyte differentiation occurs exclusively in the gastric region (David & Gierer, 1974; David & Challoner, 1977). In addition, the synthetic neuropeptide head activator (Schaller & Bodenmüller, 1981) was shown to cause commitment of stem cells to the nerve pathway (Holstein et al., 1986).

In total animal extracts of the sea anemone Anemonia sulcata and the hydroid Hydractinia a protein with tyrosine-specific protein kinase activity, which is precipitated by anti-pp60 antibodies has been detected

Correspondence: M. Schartl, Gene Center, Max-Planck-Institute for Biochemistry, Am Klopferspitz 18a, D-8033 Martinsried, Federal Republic of Germany

(Anders *et al.*, 1984; Barnekow & Müller, 1986). In the present experiments the pp60^{c-src}-related tyrosine kinase activity was assayed in Hydra in order to identify the cell types expressing this pp60^{c-src}-related tyrosine kinase activity. Our results indicate that the pp60^{c-src}-related tyrosine kinase activity of Hydra is located mainly in head tissue (which is enriched for descendants of the I cell lineage), and is preferentially present in mature nerve cells, pointing to an evolutionarily highly conserved physiological function.

Results

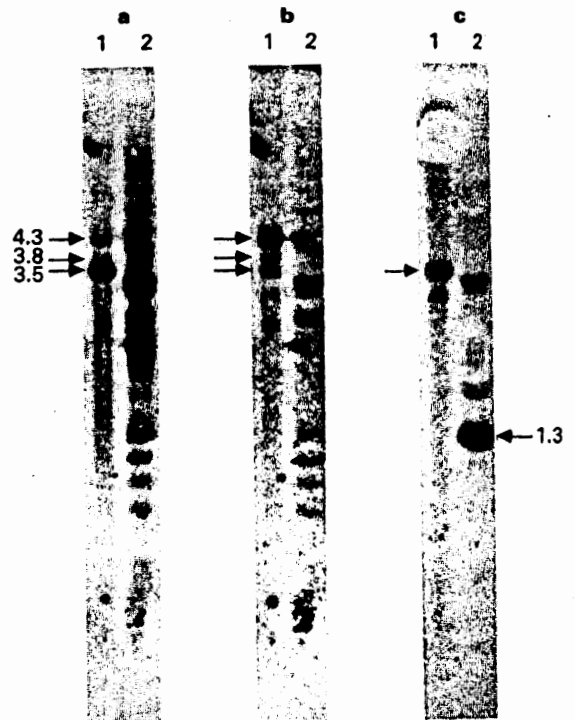### src-*related sequences and* pp60^{c-src} *kinase activity in Hydra*

In order to identify sequences in the genome of Hydra which show similarity to the *src* gene of vertebrates, a probe derived from the conserved tyrosine kinase domain of the viral *src* gene was hybridized to DNA from *Hydra viridissima*. Under conditions of moderate hybridization and washing stringency several bands were visible in Southern blot analysis (4.3, 3.8 and 3.5 kb in PstI digest; Figure 1a, lane 1) which were also detected — with varying intensities — when the filter was washed at relatively high stringency (Figure 1b, lane 1). Reprobing the filter with a genomic fragment from the kinase domain of the fish c-*src* gene (corresponding to the 5' half of the v-*src* probe) also reveals the 3.5 kb band (Figure 1c, lane 1), indicating that the coelenterate Hydra does indeed contain sequences similar to the *src* gene of vertebrates. Compared to the signals demonstrated by fish DNA, which gives numerous hybridizing bands at reduced stringency (Figure 1a, lane 2), many of which wash away at higher stringencies (Figure 1b, lane 2), the banding pattern of Hydra DNA is far less complex, and is not markedly changed in band number at higher washing stringency.

Using the v-*src* probe for hybridization on Northern blots of Hydra total RNA and poly A$^+$ RNA, a faint band of 2.5–3 kb was detected at low stringency (hybridization conditions: 35% formamide, 42°C; washing conditions: 50°C, 2 × SSC; data not shown).

To investigate the presence of a protein in Hydra with a pp60^{c-src}-related tyrosine-specific protein kinase activity we used the *in vitro* protein kinase assay (see Materials and methods). The antisera used have been shown previously to precipitate specifically pp60^{c-src} in organisms as varied as sponges and man (Barnekow & Schartl 1984; Barnekow & Bauer 1984). Immunoprecipitation from total cell extracts of *Hydra attenuata* and *Hydra oligactis* with two different anti-pp60 TBR-sera followed by the protein kinase assay revealed a significant kinase activity in these animals (see further as follows). Two-dimensional phosphoamino-acid analysis showed that exclusively tyrosine residues were phosphorylated (Figure 2).

Tyrosine kinase activity in a separated interstitial-cell lineage fraction

By physically purifying cells of the I cell lineage (I cells, nematocytes, nerve cells, gland cells and their appropriate precursors) we were able to test whether this mixed cell population contributed significantly to the tyrosine


Figure 1 Southern blot analysis of Hydra genomic DNA for the presence of c-*src*-related sequences. PstI-digested DNA of *Hydra viridissima* (lane 1) and of the teleost fish *Xiphophorus helleri* (lane 2) was hybridized to the viral *src* kinase domain probe at reduced stringency (see Materials and methods) and washed at 60°C, 1 × SSC (a, exposure 12 h, with intensifying screen). The filter was then washed at higher stringency (0.5 × SSC, 63°C) and re-exposed (b, exposure 3 days, with intensifying screen). The probe was stripped, and the filter was rehybridized to a probe that represents part of the kinase domain of the fish c-*src* gene (c). Note the presence of a 3.5 kb band in Hydra DNA in a, b and c

kinase activity detected in the intact animal. Hydra were enzymatically dissociated into single cells (see Materials and methods) and the interstitial cell lineage purified by repeated 1*g* sedimentation out of the total cell population (Figure 3). This cell fraction demonstrated a significant tyrosine kinase activity (data not shown). This result was confirmed with three different TBR-sera, indicating that the cells of the interstitial cell lineage are a major source of the pp60^{c-src}-related tyrosine kinase activity in Hydra.

Head tissue exhibits a higher kinase activity than gastric tissues

Since the nerve cell density is much higher in the head tissue than in gastric tissue (Bode & David, 1978) we
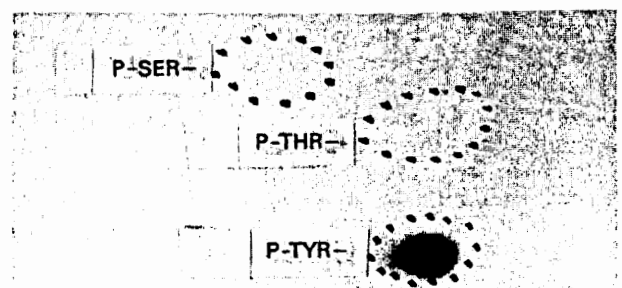
Figure 2 Phosphoamino-acid analysis of the IgG heavy chain from immunocomplexes following the kinase reaction; P-SER phospho serine, P-THR phosphothreonine, P-SER phospho serine
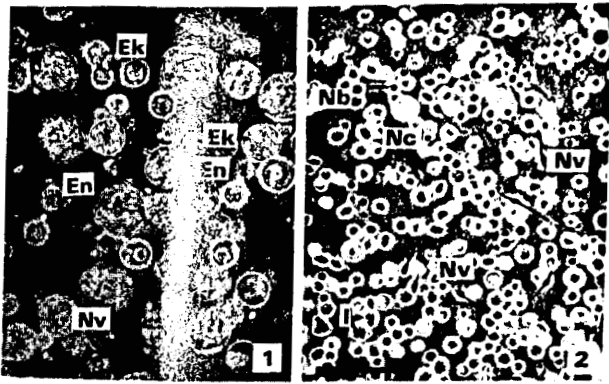
Figure 3 Cell suspensions of (1) total animals and (2) highly enriched interstitial cell lineage. Ek: ectodermal epithelial cell; En: endodermal epithelial cell; I: interstitial stem cell; Nb: nematoblast; Nc: nematocyte; Nv: nerve cell. Bar represents 100 μm

investigated whether head and gastric tissue differ in their tyrosine kinase activity. Whole budless Hydra ($n = 800$) were cut into a head fraction (hypostome and tentacles) and a gastric fraction (including peduncle and pedal disc). The specific tyrosine kinase activity (measured by the incorporation of gamma-[^{32}P]ATP into IgG heavy chain, 53K) in head tissue was almost three times higher than in the gastric tissue (Table 1; Figure 4, lanes 1–3).

Although the absolute number of nerve cells in the head does not significantly differ from the gastric region (1200 vs 1600), the nerve cell density of the head is three times higher than in the body column. In the head the ratio between nerve cells and epithelial cells increases from 0.1 to 0.3 and the number of nerve cells per μg protein increases from 39 to 133 (Table 1). These data show that the tyrosine kinase activity parallels nerve cell density.

Tyrosine kinase activity is not due to nematocytes

Since head tissue also contains a large number of mature nematocytes in the tentacles, the enhanced tyrosine kinase activity could be also due to the presence of nematocytes. For that reason nematocytes were isolated from cell suspensions of total animals in a Percoll density gradient and analyzed for kinase activity. The pellet containing mainly intact nematocytes and some nematocysts (stinging organelles of nematocytes) was completely negative in the protein kinase assay, while the buoyant fraction, consisting of all the other cell types, displayed a kinase activity comparable to whole intact animals (Figure 4, lanes 4 and 5).

Tyrosine kinase activity in a mutant strain containing temperature sensitive interstitial cells

In order to further test whether the high tyrosine kinase activity in Hydra can be attributed to particular descendents of the interstitial or the epithelial cell lineages, we

used a mutant strain (designated sf-1; Sugiyama & Fujisawa, 1978) having temperature sensitive interstitial cells. When cultured at 18°C, the sf-1 polyps contain normal cell numbers. When the temperature is raised to 25°C, over 90% of the interstitial stem cells are eliminated within 24 h (Marcum et al., 1980). Nematoblasts are eliminated within 48 h (Terada et al., 1988). All other I cell descendents (nematocytes, neuroblasts, nerve cells and gland cells) decrease in number more gradually over one week. The number of cells of the epithelial cell lineage is not affected under these conditions.

We measured the pp60$^{c\text{-}src}$ tyrosine kinase activity in animals cultured for 2, 3 and 7 days at the non-permissive temperature of 25°C. Animals after 2 and 3 days at the non-permissive temperature exhibited no change in kinase activity (data not shown), indicating that I cells and nematoblasts do not contribute considerably to the kinase activity. In animals cultured for 7 days at 25°C, no interstitial cells, nematoblasts or nematocytes could be found and the number and density of nerve cells decreased substantially from 2300 to 300 per animal and from 55 nerve cells/μg protein to 10 nerve cells/μg protein, respectively (Table 2). However, in animals at the permissive temperature (18°C) the pp60$^{c\text{-}src}$ tyrosine kinase activity was comparable to wild type Hydra, in that it was reduced to one third in animals cultured at 25°C for 7 days (Table 2; Figure 4, lanes 6 and 7). Since the number of epithelial cells in head and gastric tissue does not change significantly in such animals, we conclude that the loss in pp60$^{c\text{-}src}$ tyrosine kinase activity is caused by a loss in the interstitial cell line, and most probably by a loss in nerve cells.

As a control, kinase activity was determined in wild type animals cultured for 7 days at 25°C. The activity in
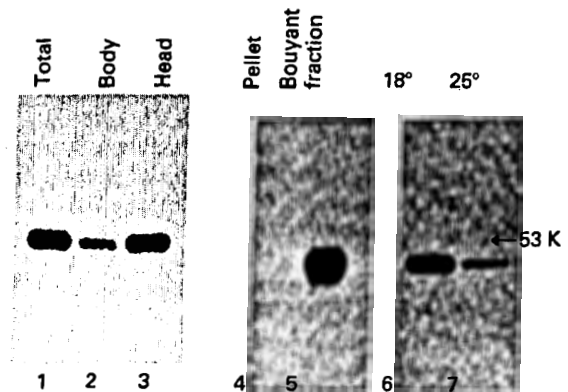
Figure 4 Protein kinase assay of cell extracts from Hydra. (1) Total extract of wildtype Hydra. (2) Body column fraction. (3) Head, hypostome and tentacle fraction. (4) Pellet containing pure isolated nematocytes. (5) Buoyant fraction, containing the remaining cells of the animals. (6) Hydra mutant sfl after 7 days at permissive temperature (18°C). (7) Hydra mutant sfl after 7 days at non-permissive temperature (25°C)

Table 1 Spatial distribution of pp60$^{c\text{-}src}$-related kinase activity in Hydra

Tissue	Protein (μg)*	Nerve cells*	Nerve cells/μg (n)	Kinase activity (cpm/mg)
Gastric region	41	1600	39	800
Head	9	1200	133	2200

* Values are given per single animal

Table 2   pp60^{c-src}-related kinase activity in a temperature sensitive mutant of Hydra

Tissue	Protein(μg)*	Nerve cells(n)*	Nerve cells/μg	kinase activity (cpm/mg)
sf-1 (18°C)	42	2300	55	1680
sf-1 (25°C)	30	300	10	540

* Values are given per single animal

these Hydra was unchanged compared to animals at 18°C (data not shown).

The Hydra c-src gene does not encode a neuronal transcript form analogous to that found in vertebrates

In vertebrates, the neuron-specific expression of the c-src gene is associated with a novel transcript form, arising through the differential splicing within neural tissue of a mini-exon of 18 basepairs located within the intron separating exons 3 and 4 (Martinez et al., 1987; Levy et al., 1987; Raulf et al., 1989). Both src-related cDNA and genomic clones have been isolated from Hydra attenuata, and found to encode a protein of similar size and with 64% sequence similarity to chicken pp60^{c-src} (T.G.C. Bosch and R.E. Steele, submitted). Oligonucleotides were prepared which span the region within the Hydra sequence analogous to the exon 3/exon 4 boundary of the vertebrate c-src genes. PCR amplification of both Hydra genomic DNA and cDNA gave an identical band of 157 basepairs (Figure 5), demonstrating that the Hydra genomic locus does not contain an intron within this region.

Discussion

We have analyzed a src-like protein tyrosine kinase in the coelenterate Hydra. Southern blot hybridization experiments have indicated that in this phylogenetically ancient organism, sequences related to the kinase domain of the c-src gene of higher vertebrates are present. This is in agreement with data that show src-like genes appearing first during phylogenesis in the most primitive metazoans — the sponges (Barnekow & Schartl 1984; Schartl & Barnekow 1982). In vertebrates, several src-like protein kinases have been found which make up a multigene family of at least seven structurally closely related genes (Hunter 1987a,b). This situation is obvious from the multiple bands that appear under conditions of reduced stringency when DNA of any vertebrate is hybridized to a probe from the conserved domain of one of the family members (see also Figure 1). Our experiments with Hydra indicate that the src gene family has fewer members in early metazoans, probably consisting of one or two genes. The fact that the protein with tyrosine kinase activity from Hydra is precipitated by antisera which are specific for the c-src gene product in birds and mammals, and which do not crossreact with other members of the multi-gene family, tempts us to assume that the c-src gene represents the ancestral progenitor gene for the whole gene family.

In vertebrates, a function for pp60^{c-src} has been suggested in nerve cell development (Maness, 1986) and/or in signal transduction in mature neurons (Walaas et al., 1988). The first organisms in evolution that have a cell

type which is morphologically and functionally equivalent to vertebrate nerve cells are the coelenterates. Our results clearly show that the tyrosine kinase activity in Hydra can be attributed to the nerve cell population. Although our evidence for this conclusion is indirect, several lines of evidence suggest that nerve cells are responsible for the tyrosine kinase activity in Hydra.

We could demonstrate that the tyrosine kinase activity is preferentially expressed in the interstitial cell population. Highly enriched interstitial cells show a tyrosine kinase activity comparable to that of whole animals. Within the interstitial cell population stem cells and differentiating nematocytes can be excluded by the fact that both cell types are absent in the head tissue which is enriched for the kinase activity as compared to the gastric region. In addition, these cell types are eliminated quickly in the temperature sensitive mutant without any noticeable effect on kinase activity. Mature nematocytes, which are also present in high numbers in head, can be excluded since isolated nematocytes showed no detectable tyrosine kinase activity. In animals of the temperature sensitive mutant strain sf1 which have been depleted of their interstitial cell popu-
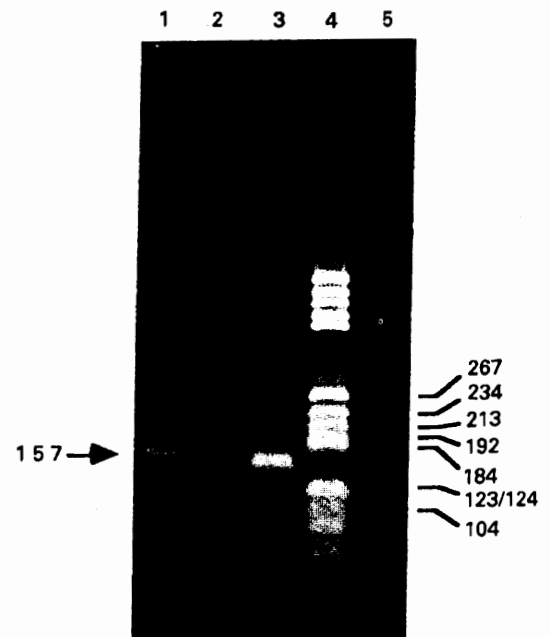
Figure 5 PCR analysis of the Hydra c-src genomic locus. A pair of oligonucleotides were prepared based upon the sequence of the Hydra stk (src-type kinase) gene [T.G.C. Bosch and R.E. Steele, submitted] which span the region of the vertebrate exon 3/4 boundary and define a 157 basepair sequence. Oligonucleotides: HY-A: (sense)5′ TTT GTC GCA CTT TAT GAT TAC GA 3′, HY-B: (antisense) 5′ GCA ACA TAA GTA GTA CTA GGA ATG TA 3′. Hydra genomic DNA or stk cDNA (kindly provided by T.G.C. Bosch) were subjected to 40 rounds of PCR amplification [92°C, 60 sec; 55°C, 60 sec]. Lanes: (1) Hydra viridissima genomic DNA; preparation 1 (0.2 μg): (2) Hydra attenuata stk cDNA (0.1 ng): (3) Hydra viridissima genomic DNA; preparation 2 (0.2 μg): (4) pBR322/HaeIII marker: (5) H$_2$O control. The PCR amplification product of 157 basepairs is marked by the arrow

lation by incubation at the non-permissive temperature, there is a drastic decrease in the tyrosine kinase activity. This decrease is caused by the loss of interstitial cells and the concomitant loss of descendent cells which differentiate from interstitial stem cells. The decrease in kinase activity parallels the loss of nerve cells, indicating that it is the nerve cells which preferentially express the pp60^{c-src} kinase activity.

Although we were not able to directly exclude gland cells, which also derive from the I cell lineage, it is very unlikely that they contribute considerably to the total kinase activity. Gland cells exhibit a graded distribution along the body column with maximum density in the upper region of the body below the head. They are scarce in head tissue (reviewed in Schmidt & David, 1986), and are therefore not expected to contribute to the enhanced tyrosine kinase activity found in the head fraction. The total gland cell population is maintained primarily by proliferation rather than by an input via differentiation from I cells. They are eliminated more slowly from sf-1 animals at the non-permissive temperature (Terada et al., 1988) than all other I cell lineage cells, and therefore are also unlikely to account for the marked decrease in pp60^{c-src} kinase activity.

The data obtained from the experiment with the temperature sensitive mutant of Hydra also demonstrate that the epithelial cells do not significantly contribute to the tyrosine kinase activity in Hydra. Since the number of epithelial cells and their specific protein content do not change after a temperature shift from 18° to 25°C, it is clear that the drastic reduction in tyrosine kinase activity is not due to the epithelial cells.

The fact that the tyrosine kinase activity in mutant animals drops only slowly and incompletely following shift to the non-permissive temperature, while differentiation of new nerve cells ceases abruptly, suggests that the tyrosine kinase activity is a property of mature nerve cells in general rather than being restricted to newly differentiating nerves. This proposal is also supported by the spatial pattern of tyrosine kinase activity in Hydra where the head, containing predominantly mature cells, shows a higher kinase activity than the gastric region.

At the moment we cannot distinguish whether the src-like protein kinase is present in all nerve cells or only in specific subpopulations, which can be characterized by means of differing neurotransmitters (van Grimmelikhuizen et al., 1982) and monoclonal antibodies (Dunne et al., 1985; E. Hobmayer et al., in preparation). We are also unable at the present time to exclude a low level of expression in non-interstitial-lineage cells. This can only be addressed in future experiments employing RNA in situ hybridization histochemistry following cloning of the Hydra src-related gene(s).

Due to the inability to metabolically label these organisms to sufficient levels to allow immunoprecipitation, it is unknown if Hydra also expresses a neuronal form of pp60^{c-src}. However, we were able to show that the Hydra genomic c-src locus exon/intron configuration differs to some extent from that of the vertebrate c-src genes, discounting a differential splicing event strictly analogous to that observed in vertebrates. This therefore suggests that the vertebrate neuronal insert within pp60^{c-src} is not a prerequisite for neural-specific expression, but is more likely involved in some as yet undefined vertebrate-specific nerve function.

The conservation of expression of the c-src gene in nerve cells during phylogeny points to a very basic function for this gene within this particular cell type. The nerve cells of Hydra are organized in a simple network and are clearly involved in signal transmission. They have a primitive type of synapse, and synaptic vesicles have also been found (Westphal, 1973). How this structural conservation relates functionally to the biochemical conservation of a src-like protein tyrosine kinase remains to be determined.

Materials and methods

Strains and culture conditions

Hydra viridissima, *H. oligactis*, *H. vulgaris* (*attenuata*), and *H. magnipapillata* strain 105 and sf1 were cultured in a modified Loomis and Lenhoff medium (M-solution) (Loomis & Lenhoff, 1956) at 18°C, fed daily with freshly hatched *Artemia* nauplii and washed 1 and 8 h later. Animals were selected from the culture 24 h after last feeding. For experiments in which the pp60^{c-src}-related tyrosine kinase activity was assayed in various regions of the body column only budless animals were selected. Such animals contain about 10 000 epithelial and 50 000 total cells.

The mutant sf1 of *Hydra magnipapillata* has temperature sensitive interstitial cells (Sugiyama & Fujisawa, 1978). Animals cultured at 18°C contain normal levels of interstitial cells and their descendents, whereas animals cultured at 25°C lose their interstitial cells within 1 day (Marcum et al., 1980).

Preparation of cell suspension from Hydra tissue

Suspension of dissociated cells were prepared by shearing large numbers of Hydra in isotonic cell culture medium as previously described (Gierer et al., 1972) or by enzymatic digestion with pronase B from *Streptomyces griseus* (Serva, Heidelberg, FRG.) at a concentration of 50 DMC-U ml^{-1} (in Hydra cell culture medium, Gierer et al., 1972) and 5×10^8 Hydra cells/ml at 18°C for 4 h.

Isolation of nematocytes

Intact nematocytes were isolated by centrifugation through a self-forming Percoll density gradient. One ml of dissociated cells (5×10^8 cells) was layered on 20 ml of 50% Percoll in Hydra cell culture medium and centrifuged at 12 000g at 18°C in a HB4 rotor (Sorvall) for 15 min. Under these conditions only nematocytes enter the gradient; all other cells float (buoyant fraction). Following centrifugation the nematocytes form a dense pellet which contains approximately 80–90% of all nematocytes in the original suspension.

Separation of interstitial cells from epithelial cells

Hydra were enzymatically dissociated to single cells by pronase B treatment. Cells of the interstitial cell lineage, i.e. stem cells, nematocytes and nerve cells were then enriched by sedimentation at 1g for 15 min in a 15 cm column filled with 2×10^7 cells/ml. The top one-third of the suspension was removed and submitted to a second cycle of sedimentation. By this method a significant enrichment of interstitial cells and I cell descendents was obtained (Figure 2).

DNA probes

All probes were separated from vector sequences after appropriate restriction enzyme digestion by low-melting-point agarose gel electrophoresis and further purification through NACS-columns (BRL, Eggenstein, FRG). The following fragments were used: (1) the 600 bp PstI fragment F of pSRA-2 (De Lorbe et al., 1980) encompassing most of the conserved tyrosine kinase domain of the viral src gene of Rous sarcoma

virus, and (2) the 600 bp PstI/XbaI fragment of the Xiphophorus genomic clone X*src* 19-4 (Robertson *et al.*, submitted) containing most of exon 8 and all of exons 9 and 10 of the fish c-*src* gene.

Southern blot analysis

High molecular weight DNA (>70 kb) from pooled total Hydra or from testes of *Xiphophorus helleri* was prepared according to the method of Blin and Stafford (1976). 10 μg of the DNA was digested to completion with restriction enzymes under conditions recommended by supplier, run on 0.8% agarose gels, and transferred to a nylon hybridization membrane (Gene Screen; NEN, Dreieich, FRG) by the alkaline transfer procedure (Reed & Mann, 1985). The filters were hybridized with approximately 1×10^7 cpm (Cherenkov) of [^{32}P]-labelled probe (using oligonucleotide priming (Feinberg & Vogelstein, 1983), specific activity usually 3–6×10^8 cpm (Cherenkov)/μg DNA). The hybridization was performed at 42°C in a buffer containing $5 \times$ standard saline citrate (SSC) and 40% formamide. Subsequent washings were performed in $1 \times$ SSC at 60°C and $0.5 \times$ SSC at 63°C.

Antisera

Sera from tumor bearing rabbits (TBR-sera) were prepared by the simultaneous injection of two different strains of Rous sarcoma virus (SR-RSV-D and PR-RSV-C) into newborn rabbits in a modification (Ziemiecki & Friis, 1980) of the procedure described by Brugge and Erikson (1977).

Preparation of cell extracts and immunoprecipitation

Samples were homogenized in extraction buffer (10 mM sodiumphosphate, 10 mM EDTA, 40 mM sodiumfluoride, 5% Trasylol, 1% Triton). The lysates were clarified as described previously (Barnekow & Bauer, 1984). An aliquot of soluble protein (0.2 or 0.4 mg) was incubated with antiserum for at least 12 h at 4°C and precipitated with the protein A-containing *Staphylococcus aureus* bacteria. The bacterial bound immunocomplexes were washed and subjected to the kinase assay (labelling for 5 min, 4°C). The protein kinase assay was carried out by a modification (Barnekow & Bauer, 1984) of the method of Collett and Erikson (1978). The reaction products were separated on 11% polyacrylamide gels. The labelled proteins were detected by autoradiography after staining, destaining and drying of the gels. For the quantitative analyses, the [^{32}P]-labelled 53K protein band was cut out of the gel, eluted, and the sample counted in a liquid scintillation counter. All immunoprecipitations were performed with at least two different TBR-sera, in all cases with the same result. Standard deviation between different measurements of the same sample was always less than 10%.

Phosphoamino-acid analysis was performed essentially as described (Barnekow & Bauer 1984).

Protein determination

Determination of protein content in cell extracts was carried out on trichloroacetic acid (TCA)-precipitated aliquots according to the method described by Lowry *et al.* (1951).

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