A Novel Invertebrate Trophic Factor Related to Invertebrate Neurotrophins Is Involved in Planarian Body Regional Survival and Asexual Reproduction

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Trophic factors are a heterogeneous group of molecules that promote cell growth and survival. In freshwater planarians, the small secreted protein TCEN49 is linked to the regional maintenance of the planarian central body region. To investigate its function in vivo, we performed loss-of-function and gain-of-function experiments by RNA interference and by the implantation of microbeads soaked in TCEN49, respectively. We show that TCEN49 behaves as a trophic factor involved in central body region neuron survival. In planarian tail regenerates, tcen49 expression inhibition by double-stranded RNA interference causes extensive apoptosis in various cell types, including nerve cells. This phenotype is rescued by the implantation of microbeads soaked in TCEN49 after RNA interference. On the other hand, in organisms committed to asexual reproduction, both tcen49 mRNA and its protein are detected not only in the central body region but also in the posterior region, expanding from cells close to the ventral nerve chords. In some cases, the implantation of microbeads soaked in TCEN49 in the posterior body region drives organisms to reproduce asexually, and the inhibition of tcen49 expression obstructs this process, suggesting a link between the central nervous system, TCEN49, regional induction, and asexual reproduction. Finally, the distribution of TCEN49 cysteine and tyrosine residues also points to a common evolutionary origin for TCEN49 and molluscan neurotrophins. © 2002 Elsevier Science (USA)

Key Words: planarian; trophic factor; regeneration; asexual reproduction; neurotrophin.

INTRODUCTION

There are several classes of trophic factors with various molecular natures, e.g., neurotrophic factors and a great variety of growth factors, among others. Moreover, the number of trophic factors has been growing. The functions of this heterogeneous group of molecules, which promote cell growth and maintain cells alive, have been extensively studied in model organisms like the vertebrate mouse, chicken, and \textit{Xenopus}, and the invertebrate \textit{Drosophila} and \textit{Caenorhabditis elegans}.

Freshwater planarians (Platyhelminthes, Turbellaria, Tricladida) are mainly known by their high ability to regenerate from a small portion of the body, according to the original polarity (Brøndsted, 1969, for a historical review). Furthermore, these primitive organisms, which have marked A/P and D/V polarities, grow and degrow depending on environmental conditions (food availability and temperature) (Baguñà and Romero, 1981; Romero, 1987; for a general review on regeneration, growth, and degrowth, see Baguñà et al., 1990). At a cellular level, both regeneration and growth/degrowth phenomena are based on the presence of a population of small undifferentiated self-renewing (stem) cells, called neoblasts, that are scattered throughout the body. Moreover, neoblasts give rise through proliferation and differentiation to all differentiated cell types both in the intact organism and during regeneration (for a general review, see Baguñà et al., 1990, 1994).

The organisms used in this study, an asexual strain of the species \textit{Girardia tigrina}, reproduce asexually by architomic fission at the postpharyngeal level, in which the fission of the “mother worm” occurs before differentiation of new organs (Hyman, 1951; for a general review, see Vorontsova and Liosner, 1960, and Palmberg, 1990). During fission, the division zone is determined by the ratios between body...
parts and has few morphologically and physiologically distinct features. Morphologically, the anterior-posterior distribution in neoblast number increases at the postpharyngeal level (Lange, 1967; Bagunia, 1976; Romero, 1987). Moreover, Stagni and Grasso (1965) noted the accumulation of neoblasts in the posterior part of the body just before division. Physiologically, several planarian species show high metabolic activity (compared with the decreasing anterior-posterior gradient of physiological activity) in the division zone (Coward and Flickinger, 1965). In addition, integrating control centers break down locally before division in this particular zone (Krichinska, 1986). To our knowledge, there is no information about the genes involved in planarian asexual reproduction, and there are no molecular markers available to monitor this process. Moreover, the association between the developmental genetic programs involved in regeneration after fission and traumatic cutting is unknown.

In freshwater planarians, a novel gene, named tcen49, is expressed in cyanophilic secretory cells from the central body region, which includes the pharynx (Bueno et al., 1996, 2001; Vispo et al., 1996). The protein encoded by this gene, TCEN 49, is a small secreted protein with a predicted Mr of 5.3 kDa that is also found in the central body region, showing extremely sharp boundaries with the anterior region, which includes the head and the cephalic ganglia, and the posterior region, which includes the tail. Neither the nucleotide nor amino acid sequence of tcen49/TCEN 49 shows significant similarity to any other reported gene or protein. The distribution of cysteine residues, however, follows the same pattern as the central stretch of the molluscan neurotrophic factor CRNF, which stands for cysteine-rich neurotrophic factor (Fainzilber et al., 1996), suggesting structural homology between these two molecules (Bueno et al., 2001). During the regeneration of traumatically cut organs that have to rebuild a new central body region and a new pharynx, TCEN 49 localizes in the area that restores these body parts before there is any morphological evidence. Nevertheless, this protein is not secreted from producing cyanophilic secretory cells until day 8–10 of regeneration, when the pharynx regains functionality.

In this paper, we analyze the role of TCEN 49 in planarian central body region survival and its involvement in asexual reproduction. We show that TCEN 49 is not necessary for the determination of the central body region, but for central body region survival when the regenerating organism starts to recover its adult functionality, functioning as a trophic factor for nerve cells. Our results also suggest a link between the central nervous system (CNS), TCEN 49, regional induction, and asexual reproduction. Finally, based on the homologous distribution of cysteine and tyrosine residues between TCEN 49 and a mollusc neurotrophin, which are also similar to EGF and EGF-like repeats from both vertebrates and invertebrates, we discuss the possible common evolutionary origin of these molecules.

MATERIALS AND METHODS

Species, Culture Conditions, and Induction of Fission

The freshwater planarians used in the study belonged to an asexual race of the species G. tigrina (Girard, 1850; De Vries and Sluys, 1991) collected near the city of Barcelona. They were maintained in spring water in the dark at 4–6°C and fed once a month with beef liver. Those selected for the experiments involving RNA interference were starved at 17 ± 1°C for at least 15 days before use. In RNAi experiments on regenerates, organisms were cut postpharyngeally (level E; Bueno et al., 1996) and kept at 17 ± 1°C.

To induce fission, 10-mm-long organisms were kept at 17 ± 1°C in petri dishes, one organism per dish to avoid the crowding factor and to stimulate fission (Vowinckel et al., 1970; Morita and Best, 1984) in day–night photoperiods (M orita and Best, 1984). They were fed once a day. When approximately one-half of the population had fissioned, within 7–10 days of fission-promoting conditions, the unfissioned flatworms were sacrificed to monitor the stages prior to fission. Fissioned organisms were sacrificed at several stages of regeneration, from day 0, i.e., a few hours after fission, to day 10.

RNA Interference

Tc49 dsRNA was synthesized following Sanchez-Alvarado and Newmark (1999), and microinjected into the mesenchyma of intact and regenerating planarians. To avoid the loss of RNA interference effectiveness, organisms were microinjected every 3–4 days from day 0 on. Intact organisms were microinjected with 52 nl of 1 μg/μl or 5 μg/μl dsRNA delivered in four microinjections in the central region; regenerating tails with 39 nl of 1 μg/μl dsRNA delivered in three microinjections and organisms under fission-promoting conditions with 52 nl of 1 μg/μl dsRNA delivered in four microinjections in the posterior region. Control organisms were microinjected with the solvent of the dsRNA, i.e., autoclaved phosphate buffer saline (PBS) 0.01 M in the same conditions. After microinjections, organisms were kept at 17 ± 1°C and sacrificed at various stages.

Implantation of Sephadex Microbeads Soaked in TCEN 49

Next, 80–100 μm Ø Sephadex G-50 microbeads (Pharmacia) were soaked in a solution containing TCEN 49 100 μM in 0.01 M PBS or only 0.01 M PBS as a negative control. The protein was obtained in a heterologous system as described by Bueno et al. (2001). Just after microbead implantation, to avoid the loss of the microbead before wound sealing, the organisms were immobilized by wrapping them in rice paper and keeping them in petri dishes, on filter papers wetted with mineral water, at 4°C for 24 h. The implanted microbead occupied one-half to two-thirds of the dorsoventral body volume, but only onethird to one-eighth of the left-right body volume, and thus it did not disrupt planarian morphology to a large extent.

In experiments of fission induction, the microbead was implanted into the tail or into the prepharyngeal region, between the eyespots and the pharynx, i.e., posterior or anterior region, respectively, one bead per organism, to both intact adult organisms far from fission body proportions and 10- to 12-mm-long adult organisms close to fission body proportions. Before implantation, the
selected organisms were kept together for 2 weeks at 17 ± 1°C in
day-night photoperiods and fed once a week to be metabolically
active but far from fission conditions (see above). After implantation,
they were isolated in petri dishes to avoid the crowding factor and
obstruction of the fission process. The organisms selected were
those in which the microbead was implanted for at least 4 days, as
the microbead is always expelled through planarian epidermis in
the end.

A control group of 20 organisms was kept in the same experi-
mental conditions except for the implantation of the microbead,
I.e., the organisms close to fission body proportions were kept
together for 2 weeks at 17 ± 1°C in day-night photoperiods and fed
once a week to be metabolically active but far from fission
conditions, after which they were kept individually in petri dishes.

In experiments of phenotype rescue after RNA interference in
regenerating tails, microbeads were implanted into the postpharyn-
geal region to avoid disrupting the prepharyngeal region, which
includes the postblastema and is very close to the regenerative
blastema and the regenerating pharynx. They were implanted in 8
days-regenerating tails microinjected with tcn49 dsRNA as de-
scribed above, 24 h before central and posterior regions started to
degenerate. A control group was kept in the same experimental
conditions except for the microbead, which was soaked in 0.01 M
PBS.

RNA in Situ Hybridization, Immunohistochemistry, and TUNEL Assay

Whole-mount planarians were hybridized as described by Bueno
et al. (1997a) using a 348-bp-long-tcn49 digoxigenin (DIG)-labeled
antisense riboprobe. Hybridization was detected by using an alka-
line phosphatase anti-DIG antibody and developed with NBT/BCIP
(Boehringer Mannheim). Images were obtained with a Zeiss Stemi
SV 6 dissecting stereomicroscope connected by a Sony video
camera to a Macintosh Centris computer running Adobe Photoshop
software.

Immunostained paraffin sections were obtained as described in
Romero et al. (1991), using the Avidin–Biotin Complex method
(ABC, peroxidase conjugated; Vector) to detect the primary anti-
bodies. Images were obtained with a Zeiss Axioskop stereomicro-
scope as described above. Whole-mount immunohistochemistry
was performed following Cebrià et al. (1997), using FITC-
conjugated antibodies (Vector) to detect the primary antibodies,
and visualized by confocal microscopy. The primary antibodies
used were TCEN49 for TCEN49 protein (Bueno et al., 1996);
TMUS-13 for muscle cells (Bueno et al., 1997b); and anti-FMRF-
amide for nerve cells (Diasorin).

Apoptotic cells were detected by the TUNEL assay on sagittal
paraffin sections. Briefly, organisms from RNAi experiments were
sacrificed and fixed in paraformaldehyde 4% on an ice-cold slide,
dehydrated in an ethanol series, and embedded in paraffin. The
10-μm sagittal paraffin sections were deparaffinated and rehydrated
through xylene and a reversal ethanol series, washed in PBS, and
permeabilized with 20 μg/ml proteinase K in PBS at pH 7.4 for 15
min at room temperature. After permeabilization, sections were
microwave irradiated at 600 W for 1 min (X5) with 0.01 M citrate
buffer, pH 3. The 3'-OH ends of DNA were labeled for 2 h at 37°C
by addition of digoxigenin 11-UTPs using the enzyme TdT and
then detected with a fluorescein-conjugated anti-digoxigenin anti-
body. Images were obtained as immunostained paraffin sections,
and the contrast was enhanced by Adobe Photoshop software.

RESULTS

TCEN49 Loss-of-Function

RNA interference in regenerating tails. RNAi experi-
ments performed on intact adult organisms did not abolish
the presence of TCEN49, as detected by immunohisto-
chemistry at either of the dsRNA concentrations tested,
i.e., 1 μg/μl and 5 μg/μl, not even in adults microinjected
every 3 days for 4 weeks, and so did not lead to complete
loss-of-function phenotype. These organisms did not show
any morphological defect (data not shown) and will not be
further discussed in this paper.

In regenerating tails, tcn49 double-stranded RNA was
injected just after amputation, when tcn49 was still not
expressed, and reinjected every 3–4 days. TCEN49 was not
detected with the antibody failing to immunohistologically
recognize TCEN49 in 32 of the 45 organisms analyzed. In
FIG. 3. The expression of tcen49 and the localization of TCEN-49 change in adult organisms committed to asexual reproduction. (A, C, and E) Whole-mount in situ hybridization with tcen49 antisense riboprobe. Anterior is to the left. Ventral view. (B, D, and F) Immunolocalization of the TCEN-49 protein on paraffin sagittal sections using a specific monoclonal antibody. Anterior is to the left, and dorsal to the top. (A, B) Stage 0. The pattern of localization of tcen49 mRNA transcripts and TCEN-49 protein is as reported for adult organisms not committed to fission (see Bueno et al., 1996, 2001). (C, D) Stage A. Arrows indicate cells expressing tcen49 mRNA in the posterior region. Arrowheads indicate areas of incipient protein expression. Note that these areas are located close to the ventral nerve chord. (E, F) Stage B. Note that the tip of the tail does not express tcen49 mRNA and that TCEN-49 protein is not detected in this small area. Abbreviations: e, eye; ph, pharynx.

FIG. 2. Tcen49 loss-of-function causes neuromuscular degeneration and apoptosis. Regenerating tails microinjected with tcen49 dsRNA (C–F, H–K, M, and N) or buffer (A, B, G, and L). Muscle cells have been immunostained using the monoclonal antibody TMUS-13 (A–F, and J), some nerve cells by using a commercial antibody against FMRF-amide (G–I, and K), and apoptotic cells have been detected by the TUNEL assay (J–N). (A–E) Confocal microscopy reconstructions. (F–N) Sagittal paraffin sections; 9–10 days of regeneration. Anterior is to the left. In confocal panels, the ventral surface is shown. In sagittal paraffin sections, dorsal is to the top. (A) Anterior region (new head) of a control organism. Note the subepidermal pattern of muscle fibers. (B) Central region at the pharynx level of a control organism. Note the subepidermal pattern of muscle fibers (down left corner), showing the pharynx cavity opening (arrowhead) and the dorsoventral muscle fibers (seen from the ventral top). (C) Anterior region (new head) of a tcen49 dsRNA microinjected organism. Note that the subepidermal pattern of muscle fibers is as in the control (A). (D) Central region at the pharynx level of a tcen49 dsRNA microinjected organism. Note that the pattern of subepidermal muscle fibers (down left corner) and dorsoventral muscle fibers is completely disorganized. (E) Pharynx of a tcen49 dsRNA microinjected organism. Note that the pattern of pharynx muscle fibers is normal, but that the pattern of the surrounding muscle fibers from the parenchyma is completely disrupted (upper left corner). (F) Muscle fibers from the central body region of a tcen49 dsRNA microinjected organism. Note the degeneration and fragmentation of some muscle fibers (arrowheads). (G) Nerve cells from the central body region of a control organism. Note the presence of a bipolar neuron (arrowhead). (H) Nerve cells from the central body region of a tcen49 dsRNA microinjected organism. Note the degeneration and fragmentation of some nerve cells (arrowhead). (I) Nerve cells from the pharynx of a tcen49 dsRNA microinjected organism. Note that the nerve cells are not degenerated. (J) TUNEL assay in a sagittal section of a tcen49 dsRNA microinjected organism immunostained with the monoclonal antibody TMUS-13, for muscle cells. Note the degeneration of the muscle fibers. (K) TUNEL assay in a sagittal section of a tcen49 dsRNA microinjected organism immunostained with a commercial antibody against FMRF-amide. (L) TUNEL assay in a control organism. The posterior part of the pharynx and the postpharyngeal area are shown. Note the presence of a couple of apoptotic cells (in yellow–green color for fluorescein). (M) TUNEL assay in a tcen49 dsRNA microinjected organism. Note the presence of apoptotic cells in the postpharyngeal area and around the pharynx, but not in the pharynx itself. (N) TUNEL assay in a tcen49 dsRNA microinjected organism rescued by the implantation of a microbead soaked in TCEN-49. The microbead is in a contiguous parallel section. Note the significant decrease of apoptotic cells with respect to the nonrescued organism (M). TUNEL and immunostaining images (F–K), and TUNEL and Nomarski images (L–N) have been superposed by Adobe Photoshop software. Abbreviations: ph, pharynx, ve, ventral epidermis. Scale bars: A–E, 0.05 mm; F–H, J, and K, 0.025 mm; I, 0.5 mm; and L–N, 0.5 mm.
the absence of TCEN-49, regeneration proceeded normally until day 9 of regeneration, i.e., a complete new pharynx was formed (Figs. 1A–1D). In control organisms microinjected with the solvent, TCEN-49 was first detected at day 1–2 of regeneration in the area where the new pharynx is formed, as described elsewhere for nonmicroinjected traumatically cut regenerates (Bueno et al., 1996).

However, at day 9–12 of regeneration, when the pharynx usually recovers functionality and TCEN-49 is first secreted from cyanophilic secretory cells, the organisms microinjected with tcn49 dsRNA lysed. We would like to highlight that just before lysis, the pharynx was ventrally and posteriorly displaced, and finally expelled from the planarian body (Figs. 1E–1L).

At this stage, before planarian lysis, cells and tissues from the putative central and posterior regions degenerated. To visualize this process, two molecular markers were used: TMUS-13, a monoclonal antibody specific for planarian muscle cells (Figs. 2A–2F), and a commercial antibody against FMRF-amide, specific for some planarian neurons (Figs. 2G–2I). Muscle fibers from the affected regions were completely disorganized (Figs. 2A–2E) and disrupted (Fig. 2F). Moreover, the FMRF-positive neurons from the affected regions were also degenerated (Figs. 2G–2I). Thus, degeneration involved neuromuscular cell death of these putative body regions and affected neither the corresponding pharynx cell types nor cells from the putative anterior region. The TUNEL assay revealed extensive apoptotic cell death in the putative central and posterior regions, including muscle and nerve cells, among other cell types from the planarian body (Figs. 2J–2N). Apoptotic cells in the putative anterior region and in the pharynx were detected as in controls (not shown).

**Rescuing of the RNA interference phenotype.** To corroborate that the absence of TCEN-49 accounts for the reported degeneration and apoptosis that ultimately led regenerating tails to lyse, experiments to rescue this phenotype were performed. A microbead soaked in a solution containing Tcen49 (25 organisms) or in 0.01 M PBS (25 organisms) was implanted into the postpharyngeal region of eight days-regenerating tails in which tcn49 as RNA was infected as described above. TCEN-49 was not detected by immunohistochemistry with the specific antibody in 12 of the 16 organisms microinjected with tcn49 dsRNA and implanted with a microbead soaked in PBS until day 9.5 of regeneration (Figs. 1i and 1j). Moreover, 5 of the 25 organisms lysed between days 9 and 9.5 of regeneration, as expected for organisms in which tcn49 expression has been properly inhibited and the phenotype has not been rescued. In the organisms analyzed, neuromuscular cell degeneration and apoptosis were as described for regenerating tails simply microinjected with tcn49 dsRNA (not shown; see Fig. 2 for similar images).

However, 15 of the 18 regenerating tails microinjected with tcn49 dsRNA and implanted with a microbead soaked in a solution containing TCEN-49 until day 9.5 of regeneration did not show neuromuscular cell degeneration or increased apoptosis (Fig. 2N), and a single organism lysed between days 9 and 9.5 of regeneration, in contrast with controls, in which the microbead was soaked in PBS (see above). In the rescued organisms, the cyanophilic secretory cells did not produce TCEN-49, as the expression of tcn49 was inhibited by RNAi; but instead they showed TCEN-49 from the microbead distributed throughout most of the organism, especially in and around the gastrodermis (Figs. 1K and 1L). The presence of nonrescued organisms is probably due to the location of the implanted microbead, totally or mostly inside a gut diverticulum, where TCEN-49 may be degraded.

On the other hand, TCEN-49 from the microbead was present all along the regenerate, especially in and around the gastrodermis, not just in the central region. This may be due to the high concentration of TCEN-49 used in these experiments, although this protein, obtained in a heterologous system, may also be posttranslationally modified in planarians. Therefore, the protein obtained in a heterologous system may replace the function of the endogenous protein but it may not be restricted to the central region.

**TCEN-49 in Asexual Reproduction**

**Expression of tcn49 and localization of TCEN-49 during asexual reproduction.** To analyze tcn49 expression and localize its secreted protein product, we performed whole-mount RNA in situ hybridization using a tcn49 riboprobe and immunohistochemistry on sagittal paraffin sections with the monoclonal antibody TCEN-49. The results described below were obtained by analyzing at least 10 organisms per stage.

Adult organisms committed to asexual reproduction were obtained from the unfissioned flatworms that were in fission-inducing conditions. Tcen49 expression and protein localization in intact adult organisms and regenerates derived from traumatic cutting have been described elsewhere (Bueno et al., 1996, 2001). We detected modifications in the expression of tcn49 mRNA and in the localization of the TCEN-49 protein before fission. Tcen49 mRNA expression and its protein product were found in the same regions as in intact adult organisms (Stage 0; Figs. 3A and 3B), i.e., in cyanophilic secretory cells within the parenchyma of the central body region except the pharynx and within the central body region including the pharynx, respectively. When committed to fission (Stage A; Figs. 3C and 3D), a few cells expressing tcn49 mRNA were first detected in the posterior (tail) region, close to the ventral nerve chords. We also observed the TCEN-49 protein in and around these tcn49-expressing cells, which reflects its secreted nature. After this transient stage, the tcn49 transcript was found all along the central and posterior regions, except the distal-most tip of the tail (Stage B; Figs. 3E and 3F), as was the TCEN-49 protein. After Stage B, organisms undergo fission at a postpharyngeal level, approximately at the posterior border of TCEN-49 localization in intact adult organisms. Most organisms fission at 7–10 days in fission-
promoting conditions, and 85–90% fission within 15 days of fission-promoting conditions.

After fission, the fissioned head/middle-body (including the pharynx) rebuilt a new tail. Both mRNA expression and protein localization receded from the areas close to the wound, where the new tail was formed (Figs. 4A–4D). In controls on day 1 of regeneration (organisms under fission-inducing treatment but traumatically cut at postpharyngeal level at Stage 0; Figs. 4E and 4F), both mRNA expression and protein localization were identical to those of day 1 fissionates (Figs. 4C and 4D). The fissionated tails rebuilt a new pharynx and a new head. Immediately after fission, both tcen49 mRNA expression and TCEN49 localization were detected all along the regenerate, except for the tip of the tail (Figs. 4G and 4H), as expected after prefission Stage B. On day 1 of regeneration, the amount of protein decreased, but not mRNA expression (Figs. 4I and 4J), suggesting that this mRNA was not translated (posttranscriptional regulation). In controls on day 1 of regeneration obtained as described above (Figs. 4K and 4L), mRNA expression was as reported for fissionates, all along the regenerate, but protein localization was restricted to a very small area, close to the ventral nerve cords (compare Figs. 4I and 4K, and 4J and 4L). From day 2 on, regeneration proceeded as described for traumatic cutting (Bueno et al., 1996, 2001), i.e., tcen49 mRNA expression and protein localization were restricted to the area where the new central body region was formed.

Induction of fission by TCEN49. To test whether the presence of TCEN49 in the posterior region is sufficient to induce fission and the effects of TCEN49 gain-of-function in the head and in the tail of intact adult planarians far from fission body proportions, microbeads soaked in a solution containing this molecule were implanted in the tail or the head of adult intact organisms. Intact adult organisms far from fission body proportions in which the microbead was implanted for at least 4 days in the prepharyngeal region or in the tail did not show any morphological defect after external inspection or in hematoxylin—eosin-stained paraffin sagittal sections (not shown; see Fig. 5 for similar experiments), and will not be further discussed.

The results described below for organisms close to fission body proportions were obtained by analyzing 102 organisms in which the microbead was implanted for at least 4 days: 18 controls and 22 treated organisms with the microbead implanted into the prepharyngeal region, 20 controls with the microbead implanted into the tail and 42 treated organisms with the microbead implanted into the tail.

Control organisms in which the implanted microbead was soaked in PBS did not show any morphological defect after external inspection (Figs. 5A, 5B, and 5F) or in hematoxylin—eosin-stained paraffin sagittal sections (Fig. 5E), and none of them underwent fission during the 4 days following microbead implantation. Likewise, organisms in which the microbead was soaked in TCEN49 and implanted into the prepharyngeal region did not show any morphological defect or undergo fission during the same period (Fig. 5C).

On the other hand, organisms in which the microbead was soaked in TCEN49 and implanted into the tail showed two responses during the 4 days following microbead implantation: 84.4% did not show any morphological defect or undergo fission (not shown), and 15.6% underwent fission at 36–48 h after microbead implantation (Fig. 5D). Finally, none of the 20 organisms from the control group kept in the same experimental conditions except for the implantation of the microbead underwent fission during the 4 days following isolation in individual petri dishes.

Obstruction of fission by tcen49 RNA interference. To ascertain whether the absence of TCEN49 in the posterior region is sufficient to obstruct fission, 25 organisms in fission-promoting conditions were microinjected with tcen49 dsRNA before tcen49 initial expression in the tail (before prefission Stage A) to inhibit tcen49 expression. Twelve of these organisms (i.e., 48%) underwent fission before day 15 of fission-promoting conditions, while the rest had not undergone fission by day 21.

The organisms that did not undergo fission were sacrificed at day 21. In these organisms, TCEN was restricted to the central body region, as detected by immunohistochemistry (Fig. 5G), indicating that tcen49 expression in the tail had been interrupted. On the other hand, in organisms that underwent fission, TCEN49 was detected a few hours after fission (day 0) in the fissionated tail close to the wound (Figs. 5H and 5I), in contrast with fissionated organisms not microinjected with tcen49 dsRNA (see Fig. 4H for comparison), in which TCEN49 was detected all along the fissionated tail, except the tip.

DISCUSSION

TCEN49 Is a Trophic Factor Involved in Regional Cell Survival

In metazoa, trophic factor molecules are associated with cell survival. This highly heterogeneous group of molecules also patterns structures and organs during embryogenesis and regulates neuronal plasticity. The loss-of-function experiments on regenerating tails presented in this paper, in which the lack of TCEN49 led to both muscle and nerve cell degeneration and apoptosis, together with the rescuing of the apoptotic phenotype by the implantation of microbeads soaked in TCEN49, in which the presence of the exogenous protein rescued the degeneration of the central and posterior regions, indicate that, at least during a specific period of planarian regeneration, when the pharynx begins to be functional, TCEN49 behaves as a trophic factor involved in regional neuromuscular cell survival.

TCEN49 has been identified with a specific monoclonal antibody (MAb) from a planarian MAbs library (Bueno et al., 1997b) as the first position-specific molecule defining a molecular body region in planarians (Bueno et al., 1996). The analysis of TCEN49 localization on regenerating and

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FIG. 4. The expression of tcen49 and the localization of TCEN-49 after fissioning during asexual reproduction are dynamic. (A, C, E, G, I, and K) Whole-mount in situ hybridization with tcen49 antisense riboprobe. Anterior is to the left. Ventral view. (B, D, F, H, J, and L) Immunolocalization of the TCEN-49 protein on paraffin sagittal sections using a specific monoclonal antibody. Anterior is to the left, and dorsal to the top. In fissionates and in control regenerates, days of regeneration are indicated in the top-right corner of the first column and apply for each row. (A, B) Anterior fragments of fissionates just after fission. (C, D) Anterior fragments of fissionates at day 1 of regeneration. (E, F) Anterior fragments of control regenerates cut postpharyngeally at day 1 of regeneration. (G, H) Posterior fragments of fissionates just after fission. (I, J) Posterior fragments of fissionates at day 1 of regeneration. (K, L) Posterior fragments of control regenerates cut postpharyngeally at day 1 of regeneration. Note the difference in protein localization between (J) and (L). The arrowhead indicates the area of protein detection in (L). Also note the lack of tcen49 mRNA expression and TCEN-49 detection in the areas close to the fission zone as early as day 1 of regeneration in all fissionates as well as in controls (arrows). Abbreviations: e, eye; ph, pharynx.
intact adult organisms suggests that TCEN-49 is involved in the determination and/or maintenance of anterior-posterior positional identities in planarians (Bueno et al., 1996, 2001). The loss-of-function results on regenerating tails presented here indicate that, although the central body region cannot be maintained in the absence of TCEN-49, this molecule is not involved in the determination of its most representative organ, the pharynx, as it forms as in control regenerates.

When considering TCEN-49 as a planarian trophic factor involved in neuromuscular cell survival, several features should be highlighted. First, TCEN-49 is a trophic factor for cells from the central and posterior regions of the body, but not for those from the anterior region, i.e., head and cephalic ganglia, or the pharynx. This may explain why the pharynx becomes detached from the rest of the body before the lysis of the whole organism, as the neuromuscular cells from the central body region that are located at the implantation zone of the pharynx, those holding the pharynx (Cebrià et al., 1999), enter apoptosis and die.

Second, in adult organisms not committed to fission, i.e., adult organisms, TCEN-49 is restricted to the central body region, suggesting that it does not function as a trophic factor for cells located at the posterior body region. However, its absence in the central region in tail regenerates also induces neuromuscular cells from the posterior region to enter apoptosis. This may be due to two mechanisms: (1) TCEN-49 affects other unknown molecules that are trophic factors for the corresponding cells of the posterior body region or (2) most probably, the primary function of TCEN-49 as a trophic factor is on nerve cells, including nerve cells from the ventral nerve chords, which run from the cephalic ganglia to the tip of the tail. TCEN-49 may thus behave as a neurotrophic factor. The latter hypothesis accounts for all reported results, as the disruption of the ventral nerve chords at the central body region in RNAi experiments would involve the death of most cells of this body region, e.g., muscle cells, and the body regions posterior to it, i.e., the posterior body region, thus not affecting the cells of the anterior body region. This also implies that TCEN-49 does not function as a trophic factor for pharynx nerve cells and that the pharynx innervation connects to the ventral nerve chords anteriorly to the central body region, where ventral nerve chords have not been disrupted by the lack of TCEN-49 in RNAi experiments.

Third, TCEN-49 is a trophic factor that becomes functional when the pharynx recovers its functionality. Accordingly, it keeps the nerve cells of the central body region alive when its regeneration is complete and the organs within it are functional.

The Pattern of TCEN-49 Cysteine and Tyrosine Residues Is Similar to That of Other Trophic Factors

The TCEN-49 amino acid sequence does not show similarities to any other protein known to date. However, the distribution of the cysteine residues may determine its nature and function, as a similar pattern is found in other proteins, like metallothioneins, scorpion toxin-like proteins (data not shown), EGF and EGF-like repeats of both vertebrates and invertebrates, and especially a molluscan neurotrophin (CRNF; Fainzilber et al., 1996). Moreover, CRNF, and EGF and EGF-like repeats also share a specific tyrosine residue between cysteine 5 and 6 (Fig. 6). EGF and EGF-like proteins are associated with epithelial cell differentiation and survival (Delgadillo-Reynoso et al., 1989; Tepass et al., 1990; Rashbass and Skaer, 2000), e.g., the EGF-like precursor protein from Drosophila, named CRUMBS, is involved in the establishment and maintenance of epithelial cell polarity during Drosophila development (Rashbass and Skaer, 2000). Drosophila EGF homologous sequences and neurogenic genes may also be related (Knust et al., 1987).

We would like to highlight that the cysteine pattern of neurotrophic factor CRNF, the first reported so far in invertebrates, is the most similar to that of TCEN-49 (Fainzilber et al., 1996). Neurotrophic factors are small secreted proteins that regulate differentiation, survival, neurite growth, and the plasticity of both central and peripheral neurons. In vertebrates, neurotrophic factors are thought to be complexity-regulating agents, whereas in invertebrates, the occurrence and roles of neurotrophic factors are still a topic of speculation, as no neurotrophic factor other than CRNF in molluscs has been described (Fainzilber et al., 1996). The primary role of neurotrophic factors in invertebrates may be to support axonal regeneration, thus enhancing the regenerative capacity of the nervous system in longer-lived invertebrates, like molluscs and planarians, which explains why neurotrophic factor homologues have not been found in organisms with short life spans, like C. elegans and Drosophila. Moreover, both molluscs and planarians belong to the lophotrochozoa, whereas C. elegans and Drosophila belong to the ecdysozoa, suggesting that these types of trophic factors are present in the former phylogenetic group but not in the latter.

Finally, the similarity between TCEN-49 and CRNF also suggests a common evolutionary origin for these and probably other related trophic factors, e.g., EGF and EGF-like molecules, although their pattern of cysteine residues is similar but not homologous.

TCEN-49 May Be Involved in Asexual Reproduction

The reported timing and dynamics of expression/localization of tcn49/TCEN-49 in fissionates (Figs. 4A–4D, 4G–4I) parallel those of regenerates derived from traumatic cutting (Figs. 4E, 4F, 4K, 4L, and data not shown; see Bueno et al., 1996, 2001 for timing and dynamics of tcn49/TCEN-49 localization). However, each initiates regeneration with its own particular pattern of tcn49 expression and TCEN-49 localization, which varies according to the cutting level and the physiological state of the original mother worm (adult organism not committed to fission or
FIG. 5. The implantation of microbeads soaked in TCEN 49 may induce fission, and tcn49 RNA interference may obstruct this process. (A–D and F) are live planarians. (E, and G–I) are sagittal paraffin sections, in which anterior is to the left and dorsal to the top. The asterisk indicates the site where the microbead has been implanted. (A) Control organism with a microbead soaked in PBS implanted in the prepharyngeal region. (B) Control organism with a microbead soaked in PBS implanted in the tail. (C) Organism with a microbead soaked in TCEN49 implanted in the prepharyngeal region. (D) Fissionated organism with a microbead soaked in TCEN49 implanted in the tail. (E) Sagittal paraffin section showing an implanted microbead. Hematoxylin–eosin staining. (F) Tail of alive organisms showing an
FIG. 6. The TCEN49 amino acid sequence does not show similarities to any other protein, but the distribution of the cysteine and tyrosine residues is similar to that for CRNF and other trophic factors. Aminoacidic sequence comparison between TCEN49 from planarian, CRNF from mollusc, and EGF and EGF-like repeats from Drosophila (CRUMBS protein, repeat 15), C. elegans (repeat 1), sea urchin (repeat 1), and human (repeat 7) (NCBI Accession Nos. AAD01277, P91758, NM_079756, CA61013, CAA35572, and P01133, respectively). The repeats represent most of the EGF-like repeats present within these proteins. The position number of the first and last shown amino acid is indicated within brackets. Cysteines are linked by filled boxes and the diagnostic tyrosine residue by an empty box.

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implanted microbead soaked in a solution containing FITC (green fluorescence). This control was performed to monitor the dynamics of the microbead during implantation. Similar controls were performed with the microbead implanted in the prepharyngeal region (not shown). Note that the microbead accounts for one-sixth to one-eighth of the left–right body volume, thus not excessively disrupting the planarian morphology. (G) Nonfissioned organism in 21 days of fission-promoting conditions microinjected every 3–4 days with tcn49 dsRNA. The section has been immunostained with the antibody specific for TCEN49. Note that TCEN49 is restricted in the central body part of the organism has not been complete or (2) most probably, the organism has fissioned through the postpharyngeal zone across the area of regular tcn49 expression, in the posterior part of the central region, where tcn49 expression is not completely inhibited by RNAi since it is expressed before dsRNA microinjections.

The relatively low percentage of organisms that undergo fission after the implantation of a microbead soaked in TCEN49, together with the significant decrease in organisms under fission-promoting conditions that undergo fission after RNA interference indicate that, although TCEN49 may be necessary to this process, it is not sufficient to induce fission, suggesting that other morphological and physiological conditions are required.

Classically, it has been proposed that these conditions are (1) the proportional relationship between body parts, which depends on several factors, e.g., food availability, (2) the accumulation of neoblasts in the posterior part of the body (Stagni and Grasso, 1965) and (3) a higher metabolic activity

preission stage B). This suggests a unique developmental genetic program used in regeneration after both natural fission and traumatic cutting.

The dynamics of TCEN49 at stages prior to planarian architomic fission is the first molecular evidence reported so far for fission in planarians that are committed to this process but do not show any morphological sign thereof. In particular, the change in TCEN49 localization, which expands to the posterior region at preission stages (Figs. 3A–3F), suggests that TCEN49 is involved in the fission process. When microbeads soaked in TCEN49 are ectopically placed in the tail, 15.6% of the organisms undergo fission 36–48 h after microbead implantation, while none of the control organisms do, and most organisms in fission-promoting conditions fission at day 7–10.

On the other hand, when organisms in fission-promoting conditions are microinjected with tcn49 dsRNA to generate RNA interference, the percentage of organisms that undergo fission before day 15 of fission-promoting conditions decreases from 85–90% to 45–50%. Moreover, the regenerating tails resulting from the fission process show traces of TCEN49 in the areas close to the wound. Two explanations may account for these results: (1) the interference has not been complete or (2) most probably, the organism has fissioned through the postpharyngeal zone across the area of regular tcn49 expression, in the posterior part of the central region, where tcn49 expression is not completely inhibited by RNAi since it is expressed before dsRNA microinjections.

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functions to this heterogeneous group of molecules. TCEN49 is a novel invertebrate trophic factor adding new processes (Kishida and Assai, 1984; Assai and Kishida, 1985). We conclude that TCEN49 is required to ensure the survival of the mother worm occurs at a postpharyngeal level, before the induction, and asexual reproduction.

Trophic Factors and Planarian Architonic Fission

During predivision stages, tcen49/TCEN49 was first detected in the tail region at predivision stages close to the ventral nerve chords. The CNS is associated with fission (Best et al., 1969) and regional induction processes, as shown by grafting of heads or prepharynx tissue experimentally deprived of nerve chords (Kishida and Assai, 1984; Assai and Kishida, 1985). The pattern and dynamics of expression of TCEN49 in organisms committed to fission, together with functional experiments by RNAi, suggest a link between the TCEN49 trophic factor, CNS, regional induction, and asexual reproduction.

During planarian architonic fission, the fission of the mother worm occurs at a postpharyngeal level, before the differentiation of any new organ or structure, by the contraction of muscle fibers from the fission zone that strangulates the body, including the ventral nerve chords, until fission is complete, simultaneously closing the wound (for a general review, see Palmer, 1990) with the participation of the CNS itself (Best et al., 1969). In this dramatic scenario, we conclude that TCEN49 is required to ensure the survival of the nervous system and thus the regional induction processes (Kishida and Assai, 1984; Assai and Kishida, 1985). TCEN49 is a novel invertebrate trophic factor adding new functions to this heterogeneous group of molecules.

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