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SHORT COMMUNICATION

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A cnidarian homologue of translationally controlled tumor protein (P23/TCTP)

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Abstract A protein homologous to P23, or translationally controlled tumor protein (TCTP), was cloned in *Hydra vulgaris*, the most ancient type of metazoan from which P23/TCTP has been characterized to date. *Hydra* P23/TCTP is composed of 184 amino acids and is encoded by a single mRNA of 700 bp. This invertebrate P23/TCTP is well conserved compared to those of other invertebrate and vertebrate species. Expression of *Hydra* P23/TCTP was confirmed by western blot of *Hydra* cell lysates using a polyclonal antibody against murine recombinant P23/TCTP. Spatial distribution of P23/TCTP mRNA and protein in *Hydra* was studied using in situ hybridization and immunostaining, respectively. *Hydra* P23/TCTP expression along the longitudinal body axis is regulated at both the transcriptional and the translational level. High levels of *P23/TCTP* mRNA were detected in a subpopulation of cells in the body column. In contrast, no mRNA was evident in the differentiated cells of the head and the foot regions. Coincidentally, P23/TCTP protein also concentrates to the body column, with no detectable protein in the head and foot region. However, despite the existence of *P23/TCTP* mRNA in both the ectoderm and endoderm in the body column, its protein is localized to the endodermal cells, suggesting a regulatory mechanism at the translational level. Taken together, the expression pattern of P23/TCTP in *Hydra* correlates with regions in which cell proliferation is actively occurring and its expression is excluded from regions where terminal differentiation has occurred.

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Introduction

P23 or translationally controlled tumor protein (TCTP) is a cytosolic protein that is widely expressed in a variety of species including plants, yeast, and metazoans (including mammals). Existing to a large extent in untranslated ribonuclear protein (RNP) particles, P23/TCTP was first identified in mouse (Chitpatima et al. 1988) and human tumor cells (Gross et al. 1989). As indicated by its name, early studies suggested the expression of P23/TCTP was mainly controlled at the translational level (Bohm et al. 1989; Bommer et al. 1994; Chitpatima et al. 1988). The expression of P23/TCTP has been linked with the growth state of cells in culture, as well as in plants (Bohm et al. 1989; Sage-Ono et al. 1998).

In the case of cells in culture, synthesis is greatly upregulated by serum stimulation (Thomas 1986; Thomas and Luther 1981). In contrast to the early assumption that P23/TCTP is a tumor-specific protein, more recent studies indicated that this protein is indeed ubiquitously expressed in many different tissues (Gachet al. 1999). At the same time, sequence analysis of the available P23/TCTP proteins revealed substantial conservation (Gachet et al. 1999). Taken together, these pieces of evidence suggest critical cellular functions of P23/TCTP, potentially in the regulation of cell proliferation. However the exact functions of P23/TCTP are unclear.

To better assess the potential function of P23/TCTP in cell proliferation, we cloned the homologous protein in *Hydra vulgaris*. *Hydra* is a representative of one of the earliest-arising animal phyla, the Cnidaria. The animal is organized as a gastric tube with a mouth and tentacles at its apical pole and a foot process at its basal pole. Its entire body lining is composed of an epithelial bilayer with an intervening extracellular matrix (ECM) (Bosch 1998). These epithelial cells take on different fates according to their position along the longitudinal axis of the organ-

ism. Epithelial cells in the body column are considered to be multipotent stem cells that continuously proliferate but also perform specific functions along the gastric tube (e.g. fluid and electrolyte transport, food absorption and digestion, etc.) (Bode 1996). As this dividing cell population is displaced into the head and foot poles, they cease to divide and differentiate/transdifferentiate into phenotypes specific for the head and foot regions (Bode et al. 1986). Therefore, *Hydra* provides an ideal model to study the dynamics of cell proliferation and differentiation, as well as the cellular events involved during these processes.

The *Hydra* P23/TCTP is a protein of 183 residues, with high sequence similarity to P23/TCTP proteins of other species. Both the mRNA and protein of P23/TCTP concentrate to the body column, where active cell proliferation occurs. The expression of *Hydra* P23/TCTP is also translationally regulated; its protein is only expressed in the endodermal cells, while its mRNA is expressed by cells in both the endodermal and the ectodermal layers.

Materials and methods

Molecular cloning and sequence analysis

A cDNA clone of 692 bp was isolated from a λ Zap II cDNA library of *Hydra vulgaris* in an attempt to identify a laminin homologue using an antibody to mammalian laminin B1 chain (Sarras et al. 1994). Both strands of this clone were sequenced using the dideoxynucleotide termination sequencing method with the Sequenase Version 2.0 DNA Sequencing Kit (USB). The DNA sequence was analyzed using the Macvector 6.0 software (Kodak) and hydrophilicity analysis was carried out using the Hopp and Woods method with a window size of 7 amino acids. Comparison of the protein sequence with sequences in electronic databases was performed using the gapped Blast program (http://www.ncbi.nim.nih.gov/) with default settings. P23/TCTP protein sequences of different species were aligned with default settings (Genetyx). Phylogenetic analysis was performed using maximum parsimony methods, with the branch and bound algorithm of the program PAUP 3.1.1 (Swofford 1993). The entire amino acid sequence was included in the analysis. The "protpars" matrix of PAUP was used to weight amino acid substitutions. Gaps were treated as characters and weighted as for a heavily weighted amino acid change.

Northern and Southern analysis

The 692 bp *Hydra P23/TCTP* was random primer-labeled with α^{32}P-dCTP using the NE Blot Kit (New England BioLabs). Total RNA was isolated from two-day starved *Hydra* as previously described (Sarras et al. 1994). After separation by 1.0% agarose electrophoresis containing formaldehyde, RNA was transferred to nylon membranes (S&S). A Northern blot of 5 μg *H. vulgaris* total RNA was hybridized overnight at 65°C with the radiolabeled probe at a concentration of 2×10^7 cpm/10 ml. The blot was washed to a final stringency of 0.1×SSC and 0.1% SDS at 65°C.

For Southern analysis, genomic DNA was isolated from *H. vulgaris* and was digested with *Eco*RI, *Hin*dIII or *Bam*HI. Ten micrograms of digested genomic DNA was separated by 1% agarose gel electrophoresis and transferred onto S&S Nytran membranes. Hybridization was carried out under the same conditions as described for Northern analysis.

Whole-mount in situ hybridization

The plasmid containing *Hydra P23/TCTP* was digested with the relevant restriction enzymes to generate sense or antisense probes. The linearized DNA template was then transcribed with T7 or SP6 RNA polymerase to generate digoxigenin (DIG)-labeled ribonucleotide probes (Ambion). Whole-mount in situ hybridization of *Hydra* was performed as previously described (Grens et al. 1995). Briefly, 2-day starved *Hydra* were fixed in 4% paraformaldyhyde at 4°C overnight. Fixed *Hydra* were incubated in 10 μg/ml proteinase K to increase tissue permeability, and then heat-treated at 80°C for 30 min to eliminate endogenous alkaline phosphatase activity. Hybridization was carried out in 100 μl of hybridization buffer containing 40 ng RNA probe (50% formamide, 5×SSC). mRNA was visualized using BM purple as substrate (Boehringer Mannheim) under conditions recommended by the manufacturer.

Immunofluorescence analysis

Immunofluorescence localization of *Hydra* P23/TCTP was performed as previously described (Sarras et al. 1994). Briefly, 2-day starved *Hydra* polyps were relaxed in 2% urethane and fixed with a 4% formaldehyde based fixative overnight at 4°C. Fixed animals were extensively washed with PBS to remove the fixative. After an overnight incubation with Q3, a rabbit anti-mouse P23/TCTP antibody, at a 1:200 dilution (kindly provided by U.A.B., St. George's Hospital Medical School), proteins were visualized using a 1:40 dilution of FITC-conjugated secondary antibodies (ZYMED).

Western blot

Proteins from sonicated *H. vulgaris* were resolved using SDS-PAGE (50 μg/lane) and transferred to nitrocellulose membranes (Bio-Rad), as previously described (Sarras et al. 1994). After preincubation in TTBS buffer (0.2% Tween-20, 10% fetal bovine serum, 1% BSA in PBS) for 30 min, membranes were incubated in a 1:500 dilution of Q3 antibody for 1 h at room temperature. After three washes in PBST, membranes were incubated in a 1:5,000 dilution of a secondary antibody conjugated with alkaline phosphatase (Promega). Protein bands were visualized with BM purple (Boehringer Mannheim). A cell homogenate of mouse papilloma 308 cells was included as a positive control (kindly provided by J.P., University of Kansas Medical Center).

Results and discussion

Cloning and sequence analysis of *Hydra* P23/TCTP

A cDNA fragment of 692 bp was isolated by screening a λ ZAP II cDNA library of *Hydra vulgaris* using a polyclonal antibody against the mammalian laminin B1 chain (Sarras et al. 1994). The cDNA contains an open reading frame (ORF) of 552 bp, together with a 5′-untranslated region (UTR) of 27 bp and a 3′-UTR of 110 bp. Sequence analysis revealed a putative protein product of 184 amino acids that is homologous to P23/TCTP, with a calculated molecular mass of 21×10^3. There are three conserved potential N-glycosylation sites. Multiple alignment analysis of P23/TCTP with its homologues from other species demonstrated a well conserved primary structure (Fig. 1A). Hydrophilicity analysis indicated that the protein has an overall hydrophilic property and lacks a signal peptide, which suggests an intracellular localization (Fig. 1B). Phylogenetic analysis places the

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             1                                                   50
Human        MIIYrDlish DEmFSDiYkI reIaDGLclE VEGKMVSRtE GnIDDSLIGG
Mouse        MIIYrDlish DELFSDiYkI reIaDGLclE VEGKMVSRtE GaIDDSLIGG
Yeast        MIIYkDIFsn DELlSDAYda KLvDDviy.E adcaMVnvgg dnID...IGa
Alfalfa      MlvYqDllTG DELlSDsYPy KeIenGmlWE VEGKwVtkgv vevD...IGa
C.elegans    MlIYkDIFTd DELsSDsfPm KLvDD.LVyE fkGKhVvRkE GeI..vLaGs
Hydra        MyIYncIFTG kEvFSDAYPh KLeDeGfVWv VdGKyeelad qkfDDSLfGG
Consensus    MIIY-DIFTG DELFSDAYPI KLIDDGLVWE VEGKMVSR-E G-IDDSLIGG

             51                                                 100
Human        NASA.EGpEg EGtESTVItG V..DIVMNhh LQE.TsFTKe a.yKKYIKdY
Mouse        NASA.EGpEg EGtESTVVtG V..DIVMNhh LQE.TsFTKe a.yKKYIKdY
Yeast        NpSA.EGgdD dvEEgaemvn ...nvVhsFR LQq.TaFdKk s.FltYIKgY
Alfalfa      NASA.EGgED EGvddTaVKv V..DIVdvFR LQEqpaFdKk q.FlgfvKrY
C.elegans    NpSAeEGaED dGsdehVerG I..DIVlNhk LvEmncyeda smFKaYIKKf
Hydra        NkSA.EGedD EGEaSTeyKp Vvntllra FR LeEpvtiTsl ndFKKalKKY
Consensus    NASA-EG-ED EGEESTVVKG V--DIVMNFR LQE-T-FTK- --FKKYIKKY

             101                                                150
Human        MKsiKgKLEE QrPER..VKp FmtGAaEqiK HILAn..FKN YQFFIGEnM.
Mouse        MKsLKgKLEE QkPER..VKp FmtGAaEqiK HILAn..FnN YQFFIGEnM.
Yeast        MKavKAKLqE tNPEe..Vpk FekGAqtYvK kvigs..FKd weFFtGEsM.
Alfalfa      iKlLtpKLda ekqEl..fKk hieGAt...K ylLck..lKd lQFFvGEsM.
C.elegans    MKNvidhmEk nNrdkadVda FKkkiqgwvv slLAkdrFKN laFFIGEraa
Hydra        tvNLmAKLnE sNqsR..Vav lKsklpkYaK qwaed..Fdk irvyvtEgdg
Consensus    MKNLKAKLEE QNPER--VK- FK-GA-EY-K HILA---FKN YQFFIGE-M-

             151                                   189
Human        ..NPDGMVaL ...LDYREDG VT..PyMiFF KDGLemEKC
Mouse        ..NPDGMVaL ...LDYREDG VT..PFMiFF KDGLemEKC
Yeast        ..dPDaMVVm ...LnYREDG tT..PFvaiw KhGivEEKi
Alfala       ..hdDGslVf ...ayYkdga ad..PtflyF ayaLkEiKC
C.elegans    egaenGqVai ...idYRdvd gTevPtlmlv KeaiIEEKC
Hydra        fevegtlVVi tqdvpfgEek pndkckMtvl aDsLIkEKf
Consensus    --NPDGMVVL ---LDYREDG VT--PFM-FF KDGLIEEKC
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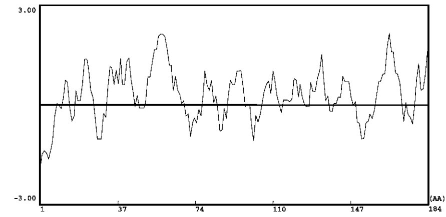
A



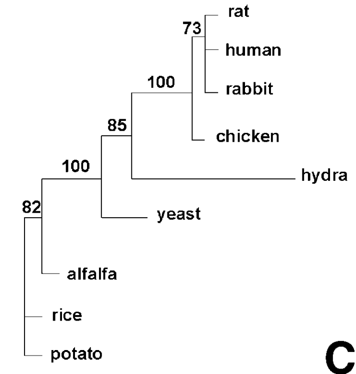


Fig. 1A–C Alignment of P23/TCTP protein sequences from different species and phylogenetic analysis. **A** Protein sequences of P23/TCTP are aligned using GCG 'Pileup' multiple sequence analysis software. The conserved amino acid residues in *Hydra* P23/TCTP are shown in *bold capitals*. Potential tubulin-binding domain is *underlined*. Potential N-glycosylation sites are in *italics*. **B** Hydrophilicity profile of *Hydra* P23/TCTP. **C** Phylogenetic analysis of representative P23/TCTP proteins. Branch length is proportional to the amount of change on the particular branch. Numbers are the percentage of 100 bootstrap replicates supporting each grouping. Genbank accession numbers of P23/TCTP sequences are: alfalfa (gi19658), *Caenorhabditis elegans* (gi2501148), chicken (gi1174624), human (gi4507669), *Hydra* (gi6094440), mouse (gi136481), potato (gi1174626), rabbit (gi6175056), rice (gi549063), yeast (gi549064)

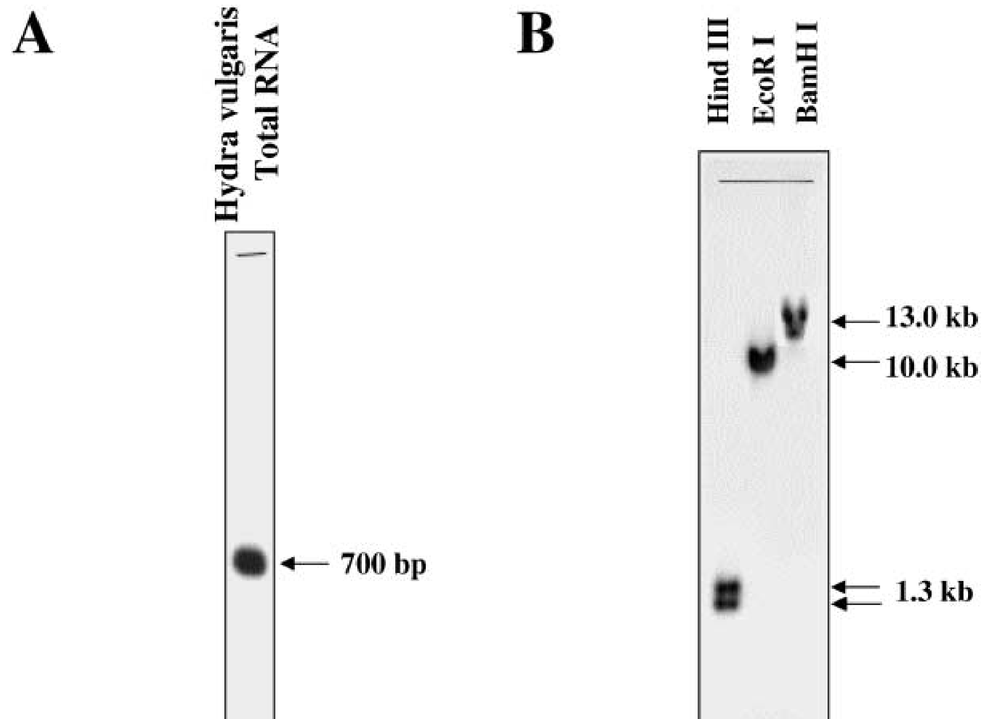


Fig. 2A, B Northern and Southern analysis of *Hydra P23/TCTP*. **A** Northern analysis of *P23/TCTP*. A single transcript of approximately 700 bp is marked by the *arrow*. **B** Southern analysis of *P23/TCTP*. Bands with their corresponding molecular weights are marked by *arrows*

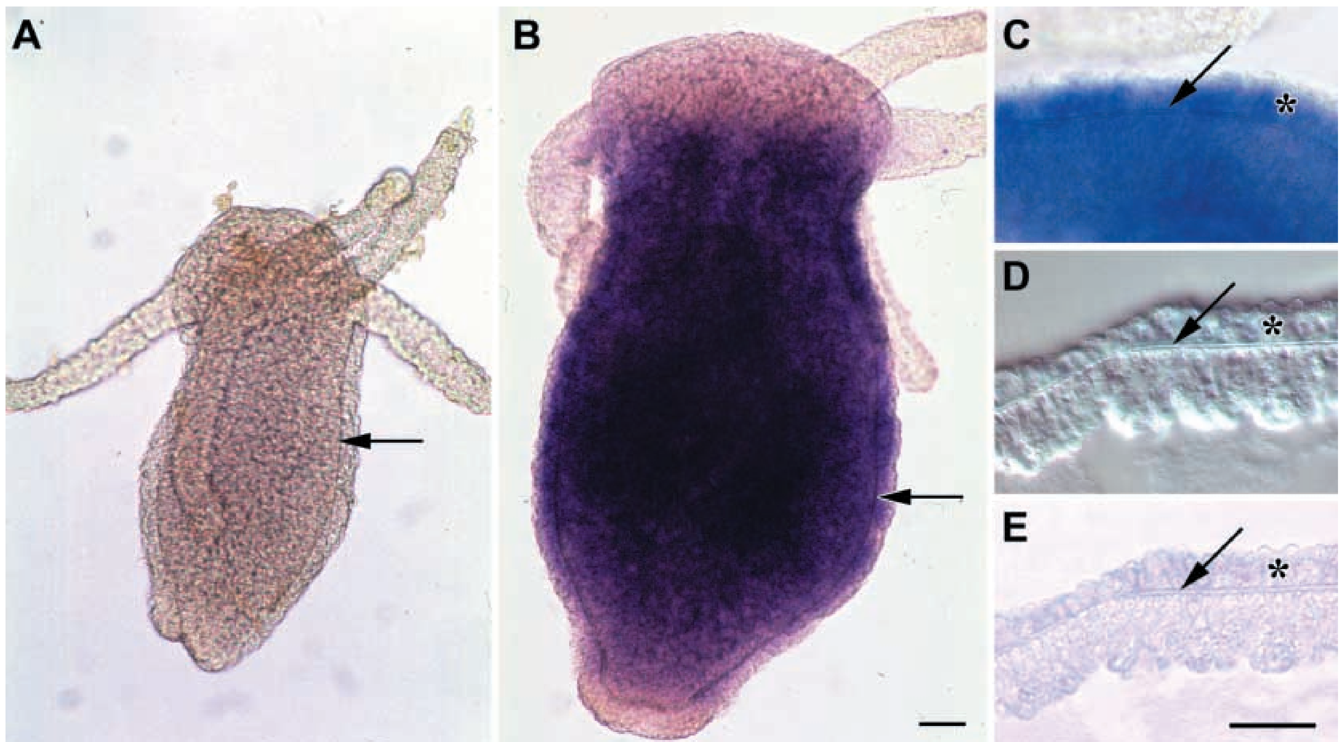


Fig. 3A–E Whole-mount in situ hybridization of *P23/TCTP*. **A** Control animal hybridized with sense probes. **B** *Hydra* polyp hybridized with anti-sense probes. **C** High magnification of the body region. **D, E** Cross section of the specimen shown in **B** that was embedded in JB-4 and subsequently sectioned to confirm the expression pattern along the body column. **D** DIC image showing detailed cell morphology. **E** Bright field image showing *P23/TCTP* mRNA within the ectoderm and the endoderm. The *arrow in all panels* indicates the extracellular matrix (ECM) that intervenes between the ectoderm (outer cell layer) and the endoderm (inner cell layer). For orientation, the ectoderm is marked with an *asterisk* in **C**–**E**. **A, B** *Scale bar* 100 µm. **C, D, E** *Scale bar* 40 µm

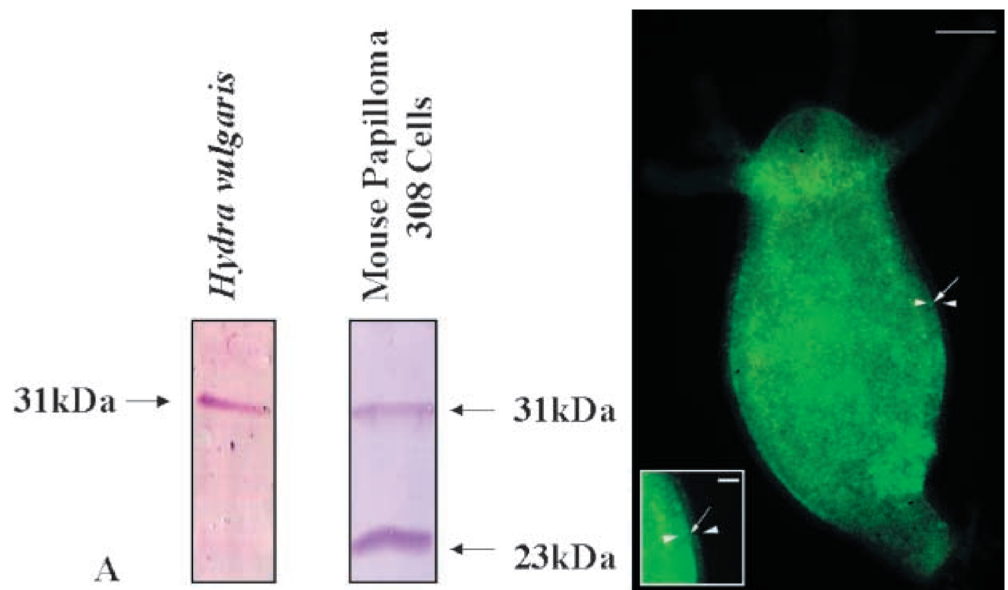


Fig. 4 Western blot analysis and immunofluorescence localization of P23/TCTP protein. *Left panel* Western analysis of homogenates from *Hydra* and a mouse tumor cell line. Protein molecular masses are indicated. *Right panel* Immunofluorescence of P23/TCTP protein. The edges of endodermal and ectodermal layers are marked by *arrowheads*. The ECM, or mesoglea, is marked by the *arrow*. The *inset in the right panel* shows a higher magnification of immunoreactive cells of the endodermal layer. *Scale bar, right panel* 300 µm: *scale bar, inset* 30 µm

protein in the position expected for a *Hydra* representative of the P23/TCTP family; this result is well supported (Fig. 1C).

Northern and Southern analysis indicated that *Hydra P23/TCTP* is encoded by a single mRNA, with an estimated size of 700 bp, which correlates well with the size of cloned cDNA (Fig. 2A). Southern analysis detected only a single band in *Bam*HI or *Eco*RI digested genomic DNA, while two bands were detected in *Hin*dIII digestion due to an internal cleavage site (Fig. 2B). Therefore, *Hydra P23/TCTP* is encoded by a single mRNA derived from a single copy gene.

Differential expression of *Hydra* P23/TCTP along the longitudinal body axis

The spatial localization of *P23/TCTP* mRNA in *Hydra* was studied by whole-mount in situ hybridization using DIG-labeled antisense RNA probe. The highest level of *P23/TCTP* mRNA was found in a subpopulation of cells that belong to both the ectodermal and the endodermal layers of the body column (Fig. 3B, C). No signal was detected in the tentacles or the foot pole. Cross sections of whole-mount in situ confirmed expression in both the ectoderm and the endoderm (Fig. 3E). The specificity of signal was confirmed with sense RNA probes (Fig. 3A). In *Hydra*, active cell proliferation is observed in the body column, while no cell division occurs in either the tentacles of the head or the basal disk of the foot process (Bode et al. 1986). As a result of this cell proliferation event, cells in the body column continuously move to the head and the foot poles. To maintain the homeostasis of the whole organism, the cells at the tips of both poles are constantly shed. Given that P23/TCTP in mammalian cells is closely related to cell proliferation, the localization of *Hydra* P23/TCTP to the active cell proliferation region supports a similar relationship in this invertebrate. Since the cells in the head region or the foot pool are derived from the body column, this differential spatial distribution of *P23/TCTP* mRNA also suggests a regulatory mechanism at the transcriptional level.

To assess the protein distribution of P23/TCTP in *Hydra*, we used a polyclonal antibody raised to murine recombinant P23/TCTP protein (kindly provided by U.A.B., St. George's Hospital Medical School). This antibody recognized a protein band of approximately 31 kDa in cell homogenates from both *Hydra* and a mouse tumor cell line in western blot analysis (Fig. 4, left panel). A protein band of 23 kDa was also detected in the mouse sample. It is likely that the 31 kDa band represents a modified form of P23/TCTP. The protein localization of P23/TCTP was studied with immunostaining using this antibody. Like its mRNA distribution, P23/TCTP protein also concentrates to a subset of cells in the body column of the animal (Fig. 4, right panel). At high magnification, a diffuse signal was detected within the cytoplasm (data not shown). This intracellular localization of P23/TCTP correlates to its overall hydrophilic property and the lack of a signal peptide. Only low levels of protein were detected in the base of the tentacles and the junction between the body column and the foot pool. The tentacles, except at the base, and the foot pole were negative for P23/TCTP staining. However, in contrast to the similar expression levels of *P23/TCTP* mRNA in both the ectodermal and the endodermal cells, its protein was detected mainly in cells of the endodermal layer (Fig. 4, insert). Although this discrepancy could be caused by the post-translational modification, it certainly could also result from regulated translation, as has been indicated in previous studies (Bohm et al. 1989; Bommer et al. 1994; Chitpatima et al. 1988).

In summary, *Hydra* P23/TCTP appears to resemble mammalian P23/TCTP not only in sequence but also in its association with cell proliferation. These findings in a member of a phylum that diverged early during metazoan evolution suggests that P23/TCTP may have played a role in cell proliferation even before divergence of protostomes and deuterostomes.

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