Cell Tissue Res (1995) 280:235–242

Cell & Tissue Research

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Boundary cells of endodermal origin define the mouth of *Hydra vulgaris* (Cnidaria)

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Received: 12 September 1994 / Accepted: 16 November 1994

Abstract. We investigated morphology, dynamics and origin of cells surrounding the mouth of *Hydra vulgaris* using the monoclonal antibody L96. This antibody recognises a one cell-thick ring of endodermal epithelial cells exactly at the boundary between endoderm (gastrodermis) and ectoderm (epidermis). L96+ cells can stretch considerably without any cell rupture during mouth opening. Thus, our data prove the existence of a distinct cell population defining hydra's mouth. A model for mouth opening is proposed and the significance of L96+ cells for boundary formation between ectoderm and endoderm is discussed.

Key words: Ectoderm – Endoderm – Boundary cells – Mouth – Cell adhesion – *Hydra vulgaris* (Cnidaria)

Introduction

The cnidarians are diploblastic organisms, that is their bodies are constructed from only two germ layers, ectoderm and endoderm. The primary embryonic germ layers are defined with gastrulation, and the cnidarian gastrula is unique in that it retains a radial symmetry, which is a symmetry of the adult. The blastopore becomes the mouth, and the archenteron becomes the definitive gastrovascular cavity (coelenteron), which combines in one cavity the digestive and coelomic functions of higher organisms. Typically, the body of polyps is tubular, with an apical oral end bearing the mouth and tentacles directed upward, while the aboral, or basal end is attached to the substratum.

The mouth is a particularily interesting structure: (i) In terms of development it corresponds to the blastopore of the gastrula and represents the most apical structure of the body axis. (ii) In terms of function, it tightly seals the gastric cavity, and also extremely expands during ingestion of the prey.

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Recently the question has been raised whether a mouth per se does exist at all in cnidarians (Campbell 1985, 1987). Epithelial cells of both germ layers can form septate junctions to close the mouth. This finding suggested that hydra creates a mouth whenever needed and allows it to heal when it is no longer neccessary, rather than to maintain a differentiated mouth that can open and close (Campbell 1987). An important consequence of this model is that no definite boundary between the two germ layers would exist.

In the present study we show that the mouth of hydra is a definitive specialized structure. We identified an endodermal epithelial cell type defining the edge of hydra's mouth by using the monoclonal antibody L96. L96+ mouth cells have a distinct morphology and can considerably expand and contract during mouth opening and closure. L96+ mouth cells define the boundary between ectoderm (epidermis) and endoderm (gastrodermis) and represent the most apical structure of the body axis known so far.

Materials and methods

Animal culture

All experiments were carried out with *Hydra vulgaris* (for taxonomy, see Holstein et al 1991) using a strain which was isolated near Basel by T. Honnegger (Zürich) and cultured by the author (T.W.H.) since 1980. Animals were kept at 18±0.5°C in M-solution (Muscatine and Lenhoff 1965) and fed daily with *Artemia* brine shrimps (Loomis and Lenhoff 1956). Experimental animals were starved for 24 h before use.

Generation and specificity of monoclonal antibody L96

The monoclonal antibody L96 was raised originally against ectodermal epithelial from *Hydra magnipapillata* (strain 105) (Schmidt, Holstein, and David, unpublished), as described elsewhere (Schmidt and David, 1986). Supernatants from hybridoma cultures were used directly for antibody staining. The staining pat-

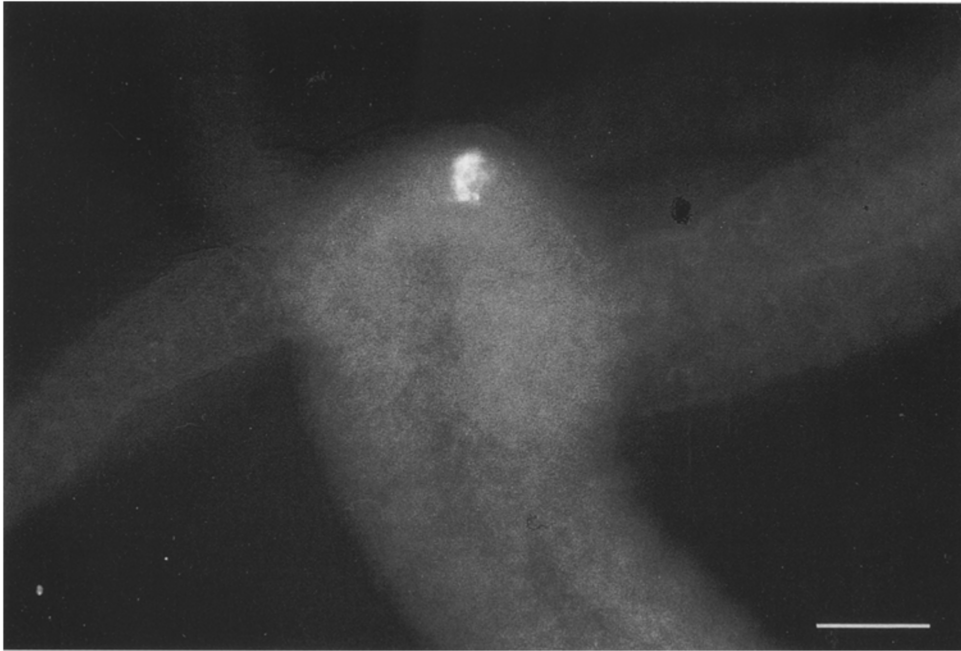

Fig. 1. Head of a polyp of *Hydra vulgaris* (Basel) stained with the monoclonal antibody L96. Note cells at the apical tip of the hypostomal dome. *Bar*: 115 μm

tern of the monoclonal antibody L96 described in this publication is strictly specific for *Hydra vulgaris* strain Basel.

Antibody staining

Polyps were relaxed in 2% urethane in hydra-medium for 1 min, fixed with Lavdovsky's fixative (50% ethanol, 4% formaldehyde, 10% acetic acid) for 24 h, washed repeatedly in PBS and incubated in undiluted solution of the monoclonal antibody L96 over night, followed by a second antibody coupled with FITC. For double staining with monoclonal antibody L96 and polyclonal antibody RFamide the polyps were prefixed in 8% formaldehyde (30 min) followed by a postfixation in Lavdovsky's fixative (20 min; modified after Koizumi and Bode 1986); thereafter animals were sequentially incubated in monoclonal antibody L96 and polyclonal antibody RFamide, followed by FITC-coupled anti-mouse antibody and TRITC-coupled anti-rabbit antibody. It should be emphasized that visualisation of L96+ antigen requires aldehyde fixation combined with ethanol and/or acetic acid treatment.

Pronase dissociation and staining

To determine the total number of L96+ cells per animal isolated hypostomes of 20 animals were dissociated with pronase E in dissociation medium (8 mg/ml; Greber et al. 1992). Dissociated living cells were centrifuged (100 *g*; 2 min), the pellet was resuspended in dissociation medium (20 μl), fixed with Lavdovsky's fixative (50 μl) and dried on one gelatine-coated coverslide. The preparations were washed in PBS and stained with monoclonal antibody L96 as described above. The total number of L96+ cells in the preparation was determined and the average number of L96+ cells per animal calculated.

Preparation of cryosections

Animals were relaxed in urethane and fixed in Lavdovsky's fixative. After washing in PBS the specimens were stepwise transferred to 10, 20, 30, 40 and 50% sucrose (cryoprotectant). Using a Reichert Frigocutt cryomicrotom, animals were quickly frozen on the quick-freeze device and 10 μm sections were prepared at –20°C. Cryosections were mounted on gelatine-coated slides, air-dried and processed for immunochemistry as described above.

DNA-measurements

Pronase-dissociated hypostomal cells (see above), were dried on slides, stained with monoclonal antibody L96, incubated thereafter in the fluorescent dye DAPI which intercalates quantitatively into DNA. DAPI fluorescence of L96+ cells, hypostomal epithelial cells and nerve cells/nematocytes (standard for G1 nuclear DNA content) was measured using a Leitz MPV microfluorometer (see Holstein and David 1990).

Microscopy and photography

All preparations were analyzed using a Leitz Dialux 20 microscope equipped with an epifluorescence attachment and Ploemopak filterblocks I2, N2 and A. Fluorescence photography was performed with Kodak Tungsten 320 (1200 ASA) or Kodak T-Max 400 (1600 ASA).

Results

L96 hypostomal cells define the site of mouth opening

The head of hydra polyps consists of an apical hypostome and a basal ring of tentacles. Monoclonal antibody L96 recognizes the apical tip of the hypostomal dome in *Hydra vulgaris* Basel (Fig. 1).

Since the hypostome of hydra is rich in sensory nerve cells (Kinnamon and Westfall 1981), we localised L96+ cells with respect to the organisation of the hypostomal

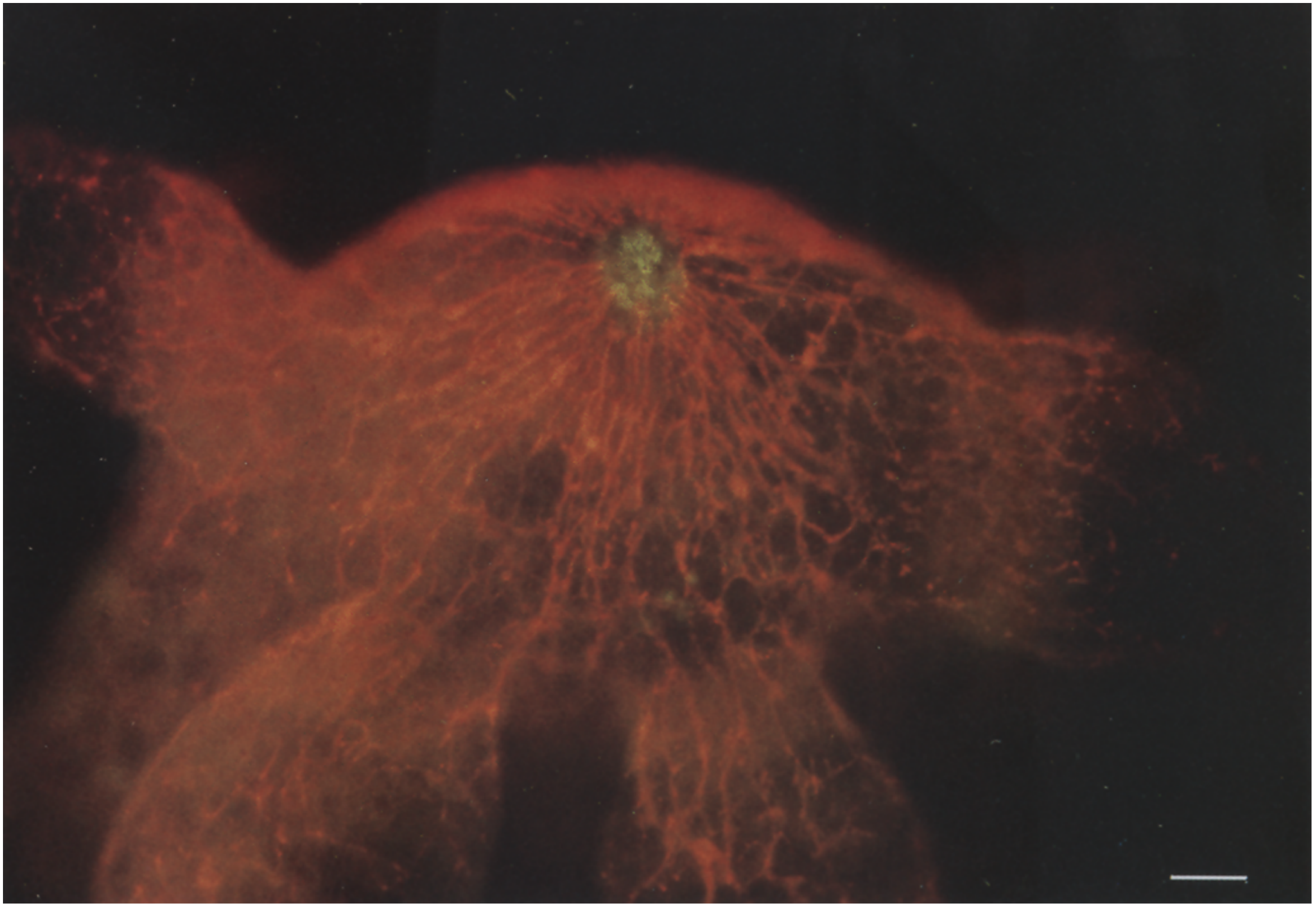

Fig. 2. Cellular organization of the hypostome. Polyps were double stained with the monoclonal antibody L96 (*green*) and anti-RFamide antibody (*red*). L96+ cells are located in the center of RFamide+ sensory neurons which run radially from the hypostomal tip to the base of the tentacles or the body column. *Bar*: 62 μm

nerve net. For that we double stained polyps with monoclonal antibody L96 and a polyclonal antibody specific for RFamide-like neuropetides (Grimmelikhuijzen et al. 1982; Grimmelikhuijzen 1983, 1985).

As shown in Fig. 2, L96+ cells were localised in the center of a radially RFamide+ nerve net. RF amide+ neurons were always excluded from this central L96+ region. This characteristic arrangement of neurons and L96+ hypostomal cells suggests that the hypostomal tip is a highly organized structure bearing a well defined site for mouth opening.

In order to investigate the potential role of the L96+ cells during mouth opening we analysed the mouth structure during feeding. Hydra were fed on *Artemia* nauplii and fixed at various stages of prey ingestion.

L96+ cells formed a thin ring around the site of mouth opening without any loss or rupture of cells (Fig. 3). Even at stages of extreme mouth opening (Fig. 3D) when the mouth reached a diameter of up to 1 mm, this ring remained intact indicating that L96+ hypostomal cells exhibit a remarkable elasticity and tensile strength. Thus, hydra polyps have a distinct mouth which is defined by L96+ hypostomal cells (see "Discussion").

L96+ cells are of endodermal origin

In order to determine from which germ layer L96+ cells derive, we localised L96+ cells in cryosections. Figure 4 shows two longitudinal sections through a hypostome with an opened and closed mouth, respectively. In animals with a closed mouth, L96+ cells seemed to be clearly located in the endoderm (Fig. 4A, C). However, in animals with an opened mouth, ectoderm and endoderm form a continuous layer (Fig. 4B, D), which makes the decision to which germ layer L96+ cells belong more difficult.

We have therefore tested the origin of L96+ hypostomal cells in polyps whose germ layers were labeled with fluorescent latex beads. By this method, 55% (±14%) of all ectodermal epithelial cells and 55% of all endodermal epithelial cells (±12%) specifically incorporate fluorescent latex beads (Technau and Holstein 1992). We removed the upper half of the animals, and allowed them to regenerate a head. On day 4 after head removal polyps were enzymatically dissociated into single cells, and the fraction of labeled L96+ cells was determined (see "Materials and methods").

We found that 63% of all L96+ cells contained beads when the endoderm was labeled prior to head removal.

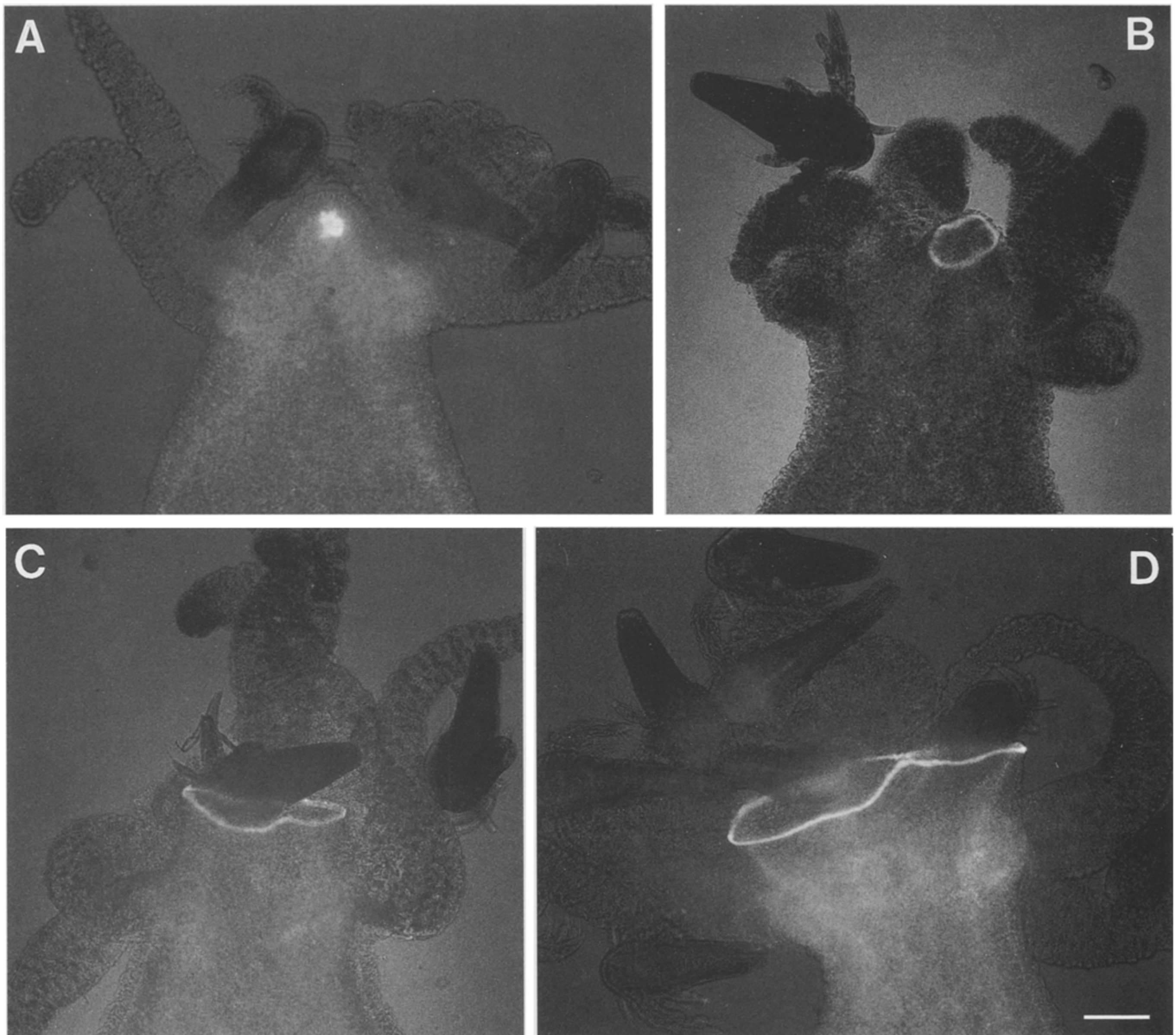

Fig. 3A–D. Mouth opening during prey ingestion. Hydra were fed on *Artemia* nauplii, fixed and stained with monoclonal antibody L96 after various stages (**A–D**) of ingestion. Even at extreme mouth opening (**D**), L96+ hypostomal cells form a narrow ring without any rupture or cell loss. *Bar*: 160 μm

By comparison, only 7% of all L96+ cells contained beads when the ectoderm was labeled; this labeling can be explained by the low rate of mislabelling (5–10%) due to the labeling technique (Technau and Holstein 1992). Thus, L96+ hypostomal cells arise from endodermal epithelial cells.

Cellular characterisation of L96+ hypostomal cells

To determine the number of L96+ hypostomal cells per animal we isolated hypostomes from 60 polyps and dissociated them with pronase E (see Materials and methods). We found an average of 11±3 L96+ cells per hypostome. The number of L96+ hypostomal cells was not significantly correlated to the number of tentacles in the head, indicating that the hypostome has a limited size regulation (see also Bode and Bode 1984).

To analyse the size of L96+ hypostomal cells we also used pronase E-dissociated cell suspensions (Fig. 5C). L96+ cells were relatively small (19.9±4.5 μm), compared to the size of ectodermal (20–25 μm) and other endodermal epithelial cells (25–30 μm) (Greber et al. 1992).

To examine the morphology of L96+ cells it was necessary to use macerated cell preparations (David 1973), which preserve the in situ morphology of hydra cells. Mostly, cells had a narrow shape (Fig. 5A, B). The cytoplasm was characterized by a number of small granules or vacuoles, clearly smaller and optically different from the nutritive vacuoles of endodermal epithelial cells

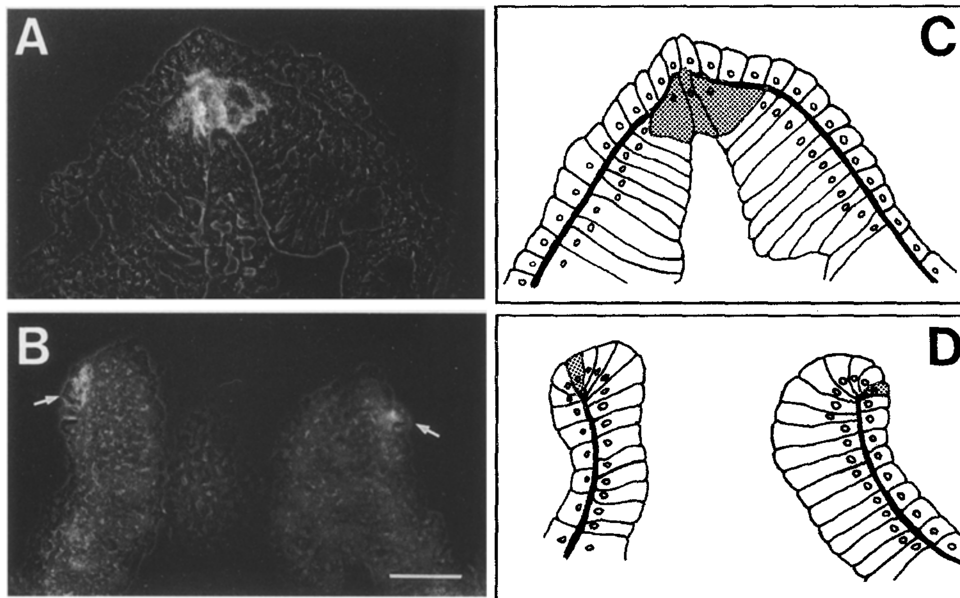

Fig. 4A–D. Localisation of L96+ hypostomal cells in cryosections. Longitudinal sections through the hypostomal tip of polpys with closed mouth (**A**) and opened mouth (**B**); *schematic drawings* (**F**, **D**) indicate localisation of the mesoglea (*thick line*), ectodermal and endodermal cells and L96+ cells (*hatched area*); *arrows* in (**B**) indicate L96+ cells (for further details, see text). *Bar*: 90 μm

(Fig. 5A). The nucleus contained a nucleolus which was much less pronounced than in normal epithelial cells; the nuclear structure was reminiscent to the morphology of G_1 nuclei of neurons and nematocytes.

In order to investigate the cell cycle position of the L96+ cells we dissociated isolated hypostomes with pronase E, fixed and double stained them with the monoclonal antibody L96 and with the fluorescent dye DAPI. DAPI intercalates quantitatively into the DNA of cells, e.g., the intensity of the emitted fluorescence is correlated to the amount of DNA. We measured DAPI fluorescence in L96+ cells and found that they had a DNA-content similar to that of nerve cells and nematocytes (Fig. 6A, B). By comparison, the majority of all other epithelial cells in the hypostome were in G_2 or S-phase of the cell cycle (Fig. 6C). Since cycling epithelial cells have no G_1 (David and Campbell 1972), we conclude that the L96+ hypostomal cells are terminally differentiated and arrested in G_1.

Endodermal epithelial cells which are arrested in G_1 comprise about 10% of all epithelial cells in the apical hypostome (Fig. 6C) containing about 150–200 epithelial cells. From that one can estimate that the majority of endodermal epithelial cells, which have been previously identified as G_1 cells (Dübel 1989) are effectively L96+ cells.

Discussion

Structure of hydra's mouth

We identified a new epithelial cell population in *Hydra vulgaris* by using the monoclonal antibody L96. L96+ hypostomal cells are terminally differentiated and arrested in the G_1 phase of the cell cycle. L96+ cells derive from the endodermal epithelial cell lineage and define a ring of about 10 cells around the mouth, just at the boundary between ectoderm and endoderm.

Heretofore, the site of mouth opening in hydra was investigated mainly on the ultrastructural level in *Hydra littoralis* (Beams et al. 1973; Westfall and Townsend 1976; Wood 1979a, b), *Hydra oxycnida* (Campbell 1987), *Hydra viridissima* (Campbell 1987), and *Hydra vulgaris* (Wood 1979a, b; Campbell 1987). These studies suggested the existence of cells at the margin of the mouth which resisted classification as either ectodermal or endodermal cells (Westfall and Townsend 1976; Wood 1979a, b). Cells visible on the outside of the closed mouth were called "lip cells" (Westfall and Townsend 1976) or "rosette cells" (Campbell 1987). Cells on the inner side of the mouth were called "transitional cells" (Wood 1979a, b) or "plug cells" Campbell (1987).

Localization and morphological features of L96+ cells indicate that they are identical to transitional cells (Wood 1979b) and plug cells (Campbell 1987). In the light of the present results, however, both denominations are no longer appropriate. L96+ hypostomal cells are clearly cells of the endodermal cell lineage; they therefore do not represent transitional types of cells between the germ layers. L96+ hypostomal cells also have a highly variable form, which typically changes from a plug-like structure in the closed mouth (Fig. 4A) to a ring-like structure in the opened mouth (Fig. 3D). Since these cells represent the boundary between both germ layers (see below), we propose to call them the "boundary cells".

How does hydra open its mouth?

Previous work has shown that epithelial cells at the apical tip can be tightly connected by septate junctions when the mouth is closed forming thereby an uninterrupted epithelium (Campbell 1985, 1987).

Based on these data, a mechanism for mouth opening was proposed where a mouth per se did not exist: The

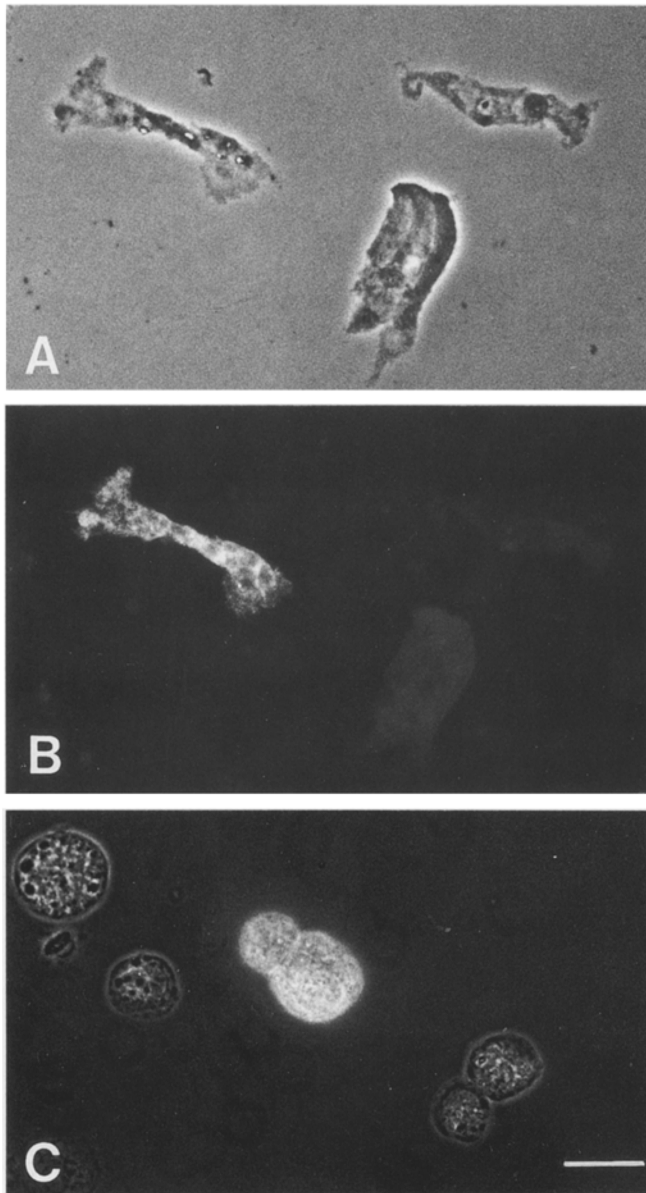

Fig. 5A–C. Dissociated hypostomal cells. Hypostomes were dissociated by maceration (**A**, **B**) or by treatment with pronase (**C**) and stained with the monoclonal antibody L96. L96+ cells are mostly narrow with small inclusions but lacking large nutritive vacuoles. Mucus cells (**A**, **B**; lower cells) and other epithelial cells (**A**, **B**; upper cells) are clearly negative. Cells lose their morphology when dissociated with pronase, but the antigen is still preserved under these conditions (**C**). *Bar*: 20 μm

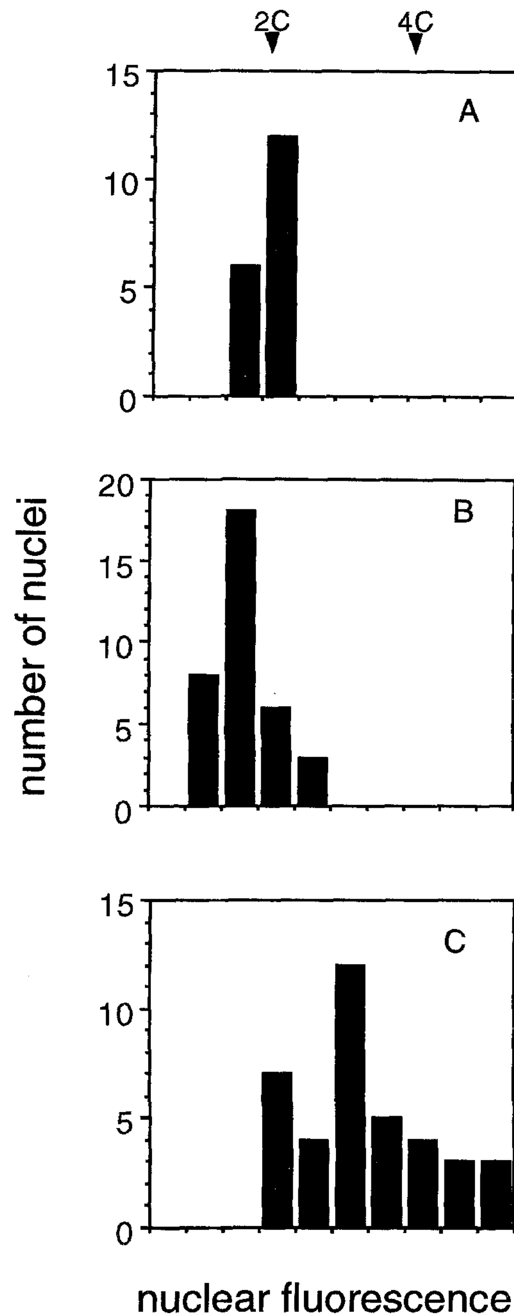

Fig. 6A–C. Nuclear DNA-content of hypostomal cells determined by microfluorometry. Isolated hypostomes were dissociated with pronase E and double stained with monoclonal antibody L96 and the nuclear fluorescent dye DAPI (see "Materials and methods"). *Abscissa* Relative DNA-content in arbitrary units of DAPI fluorescence. (**A**) Nerve cells and nematocytes used as a standard to determine G_1 position. (**B**) L96+ hypostomal cells. (**C**) Hypostomal epithelial cells (L96−)

mouth should open by a rupture in the epithelium at the point of highest tension and expand by intensive rearrangement of cells. Cells from more proximal positions should slip apically to form the margin of the mouth, which finally was estimated to be comprised by more than 100 cells (Campbell 1987). Our experiments reveal that this is clearly not the case; the margin of the mouth in *Hydra vulgaris* is comprised of about 10 cells, which can considerably expand and contract during mouth opening and closure, without any rupture of the epithelium (Figs. 3–4).

By comparing foregoing ultrastructural observations (Wood 1979a, b; Campbell 1987) with the results of the present report it is possible to outline the mechanics of mouth opening as shown schematically in Fig. 7. Mouth opening begins when axially oriented muscular processes of the ectodermal cells contract. Since L96+ cells are connected with ectodermal cells, they become pulled outwards by this movement (giving the impression that

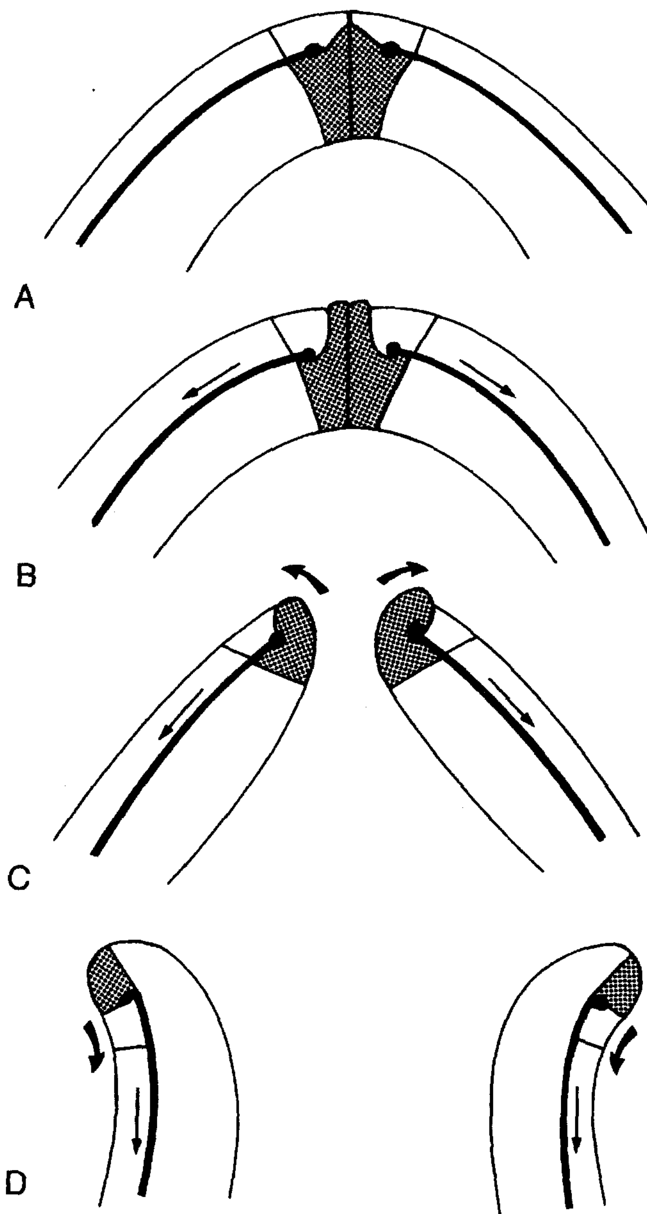

Fig. 7A–D. Model for mouth opening in Hydra. Drawings show the hypostomal dome with ectoderm, endoderm and mesogloea (*thick line*). Only endodermal boundary cells (L96⁺) (*dotted*) and the presumptive ectodermal boundary cells (*white*) are specified schematically. See text for explanation

they are squeezed between ectodermal cells; Fig. 7B). Further contraction of the ectodermal muscular processes leads to the opening of the mouth and pulls L96⁺ cells exteriorally around the end of the mesogloea (Fig. 4A, C; see also Wood 1979b, Fig. 25), which has the function of a "hinge". By this axial contraction, L96⁺ cells become stretched. When the mouth is widely open (Fig. 3C), L96⁺ cells form the edge of the mouth opening. At the extreme the L96⁺ cells can even be pulled further giving the impression that they lie in the ectodermal layer (Fig. 4B, D; see also Wood 1979a, Fig. 8).

During mouth opening cells become stretched considerably. One can calculate that in a widely opened mouth (Fig. 3A), which consists of 10 L96⁺ cells and measures about 2000 μm in circumference, a single cell (20 μm in diameter) becomes stretched by a factor of 10 (6 μm in diameter and 200 μm in length). This is not extraordinary for hydra cells, since epithelial cells of the body column and the tentacles can expand by a factor of about 8 (data not shown).

Closure of the mouth is probably the reverse process with the exception that here the endodermal cells, e.g., the L96⁺ cells are playing the active part: Their circumferentially arranged muscle fibers contract, which narrows the mouth opening until the cells at the margin meet together in the center of the hypostome. Then they form close contacts and seal the mouth by constant contraction and strong cell-cell contacts via their interdigitating processes. Thus, the function of L96⁺ cells can be described as that of a sphincter.

Cell-adhesion complexes may help to keep the mouth closed (see Campbell 1987). This is reminiscent to the ctenophore *Beroë* where mouth opening can be also accompanied by rapid changes in cell adhesion complexes (Tamm and Tamm 1991, 1993). The L96 antigen of endodermal boundary cells could be important for this specific cell-adhesion mechanism. For example, the L96 antigen could be part of heterotypic cell-cell contacts between ectodermal and endodermal boundary cells. A functional role of the L96 antigen is suggested by regeneration experiments demonstrating that L96 antigen expression was always correlated with the formation of a functional mouth (Technau and Holstein, unpublished).

L96⁺ cells define the boundary between endoderm and ectoderm

Boundaries between tissues or compartments are of general biological interest, since they reflect an interesting problem for the organism: how is a sharp boundary generated during development and how is it maintained. In amphibian gastrulation a small number of "bottle cells" are induced at the boundary between ectoderm and endoderm which initiate invagination and involution of the prospective endoderm (Hardin and Keller 1988). In the imaginal wing discs of *Drosophila* sharp boundaries are generated to form dorsal and ventral as well as anterior and posterior compartments (for review, see Ingham and Martinez-Arias 1992). The cells at the boundary, for instance, between dorsal and ventral compartments form the later wing margin and have specific identities. They are induced at the interface between dorsal and ventral cells suggesting a short-range interaction. It is therefore likely that the boundary cells serve as an organizer center in the patterning of compartments (Meinhardt 1983, 1991; Diaz-Benjumea and Cohen 1993; Couso et al. 1994; Technau and Holstein 1995).

Acknowledgements. This work was supported by the Deutsche Forschungsgemeinschaft (DFG Ho-1–3) and a scholarship of the Studienstiftung des deutschen Volkes (U.T.). We thank K. Bauer for her excellent technical assistance in rising hybridoma cell lines and C.J.P. Grimmelikhuijzen (Copenhagen) for kindly providing anti-RFamide serum.The initial experiments were carried out in the laboratory of C.N. David (München).

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