Microtubule-dependent cell cycle regulation is implicated in the G2 phase of
Hydra cells

STEFAN DÜBEL*
Zentrum für Molekulare Biologie Heidelberg, D-6900 Heidelberg, FRG

and MELVYN LITTLE
Institut für Zell- und Tumorbiologie, Deutsches Krebsforschungszentrum, D-6900 Heidelberg, FRG

*Author for correspondence

Summary

Interstitial cells of Hydra attenuata, from which nerve cells and nematocytes (stinging cells) differentiate, were arrested in either metaphase or G2 by different concentrations of the microtubule-depolymerizing agent nocodazole. At a concentration of 1.4 nM-nocodazole, a large number of cells were arrested in metaphase. However, at concentrations of 2 nM-nocodazole and above most of the cells were arrested at a distinct point in G2 several hours before mitosis. After removal of the 2 nM-nocodazole block, 75% of the cells entered the next cell cycle about 10 h later. To our knowledge this is the first time that cells have been synchronized by arresting them in the G2 phase. Visualization of Hydra microtubules with a tubulin monoclonal antibody and immunofluorescent staining showed that the very low concentrations of nocodazole used for cell cycle arrest were indeed affecting microtubule structures. Spindles and stem cell microtubules disappeared at 0.8-1 nM-nocodazole, followed by nerve microtubules (about 2 nM), cnidocil microtubules (10 nM) and finally by nematocyte microtubules (34 nM). Taken together, these data strongly indicate a microtubule-dependent mechanism of cell cycle regulation in the G2 phase.

Key words: microtubules, cell cycle, Hydra.

Introduction

The relatively simple form and small number of different cell types of the freshwater coelenterate Hydra attenuata make it an attractive organism for studying differentiation and cellular interactions. Both the body wall and the tentacles of hydra are composed of an ectodermal and endodermal epithelium separated by an acellular mesogloea. Hydra nerve cells are derived from proliferating interstitial cells that migrate in the intercellular spaces to their respective destinations. Interstitial cells can also differentiate into nematocytes (stinging cells), which, in the tentacles, are situated within pockets of terminally differentiated ectodermal epitheliomuscular cells. They are anchored to their host cells and to the mesogloea by a complex configuration of junctions (Slatterback, 1967; Wood & Novak, 1982; Campbell, 1987). The epitheliomuscular cell together with its content of nematocytes and neurones is referred to as a 'battery cell complex' (Hyman, 1940; Hufnagel et al., 1983). Movements of prey against the sensory cnidocil of the nematocytes triggers nematocyte discharge followed by concerted tentacular reflexes, mouth opening and ingestion. Sensory neurones that have been activated by cellular components released from damaged prey tissue also appear to play an important role in the feeding response. Their high concentration and complex synaptic organization around the mouth area (Westfall & Kinnamon, 1984) suggest that they are particularly important for mouth movements.

Investigations into the differentiation of interstitial stem cells to neurones and nematocytes would be facilitated if their cell cycles could be synchronized. We therefore incubated these cells with various concentrations of the microtubule-depolymerizing agent nocodazole in an attempt to arrest their cell cycles in metaphase. This drug was chosen rather than the classical mitotic poison, colchicine, for two reasons. First, colchicine appears to have only a low affinity for Hydra tubulin (Campbell, 1976); and second, nocodazole has a high affinity for the tubulins of several taxonomically distant species. Surprisingly, we found that although cells were arrested only in metaphase at the lowest effective concentrations of nocodazole, at concentrations of 2 nM-nocod-
azole and above, most of the cells were arrested at a distinct point in the G2 phase. On the basis of these results we devised an efficient cell cycle synchronization procedure that should prove useful in differentiation studies in Hydra and possibly in other primitive organisms. To determine whether Hydra microtubules were affected at these very low concentrations of nocodazole, they were visualized by immunofluorescent staining. We observed that spindle and stem cell cytoplasmic microtubules were indeed depolymerized by the concentrations of nocodazole used for cell cycle arrest. Our results strongly suggest, therefore, that the formation of a particular microtubule structure is required at a distinct point in G2 before the cell cycle can progress any further.

Materials and methods

Growth conditions

Hydra attenuata were cultured in a medium consisting of 1 mM-CaCl₂, 0.1 mM-KCl, 0.1 mM-MgCl₂, buffered with 0.5 mM-sodium phosphate, pH 7.6. They were fed daily between 9 and 10 a.m. with nauplii of Artemia salina and washed 5–6 h after feeding. The water temperature was kept at 19°C (±2)°C.

Western blotting

Total protein was prepared by sonicating hydras in ice-cold sample buffer containing 4% SDS, 1 mM-CaCl₂, 10% glycerol, 10% 2-mercaptoethanol, 0.02% Bromophenol Blue, buffered with 125 mM-trisaminomethane • HCl at pH 6.8. After an incubation of 5 min at 0°C, the homogenate was heated to 95°C for 2 min and centrifuged for 10 min at 13,000 g. SDS–polyacrylamide gel electrophoresis of the supernatant was carried out on a 12% polyacrylamide gel (Laemmli, 1970). Western blots were performed according to Towbin et al. (1979) with the monoclonal anti-tubulin antibodies DM1A, DM1B (Amersham, Braunschweig, FRG) and YOL1/34 (Camon Labor Service, Wiesbaden, FRG) diluted 1:1000 in 1% casein in PBS. After washing three times for 10 min in PBS, animals or slides were incubated for 1 h at room temperature with FITC-labelled anti-mouse or anti-rat IgG (Dakopatts, Hamburg, FRG) at a dilution of 1:50 in 1% casein in PBS. After washing twice for 10 min in PBS, the preparations were counterstained for DNA by incubation for 10 min at room temperature in a solution of 0.5 mg/l HÖECHST 33258 (Serva, Heidelberg, FRG) in PBS, and mounted in PBS/glycerol (1:2, v/v) containing 1 mg/ml 1-phenylenediamine.

Nocodazole incubations

At 5–6 h after feeding, hydras were washed free of debris and incubated at a density of two animals per ml of medium with various concentrations of nocodazole (Janssen Biochemica, Beersse, FRG). They were incubated in a 40 μg/ml 1-phenylenediamine stock solution in dimethyl sulphoxide. Controls were treated with equivalent dilutions of dimethyl sulphoxide only. The nocodazole was removed by washing three times with 20 volumes of culture medium.

Assay for metaphase arrest

After incubating hydras for 44 h with nocodazole, they were fixed in 4% formaldehyde in PBS for 30 min, stained for 30 min at room temperature in a solution of 0.5 mg/l HÖECHST 33258 and embedded in PBS/glycerol (1:2, v/v). Gastric regions were then screened for cells with condensed chromosomes using a fluorescence microscope.

Measurement of DNA in single nuclei

Upper gastric portions of steady-state hydras were macerated and their dissociated cells were fixed on microscope slides as described by David (1973). The cells were then stained for 30 min with a solution of 0.5 mg/l HÖECHST 33258 in PBS (Cowell & Franks, 1980) and mounted in this solution. The fluorescence of single interphase nuclei was determined with a ZEISS microscope photometer using the filters G365, FT395 and LP420. Cells were considered to be in the G0 or S phase of the cell cycle when their nuclear fluorescence value was smaller than the threshold \( X_{2n} = (2 \times X_{4n}) + \text{S.D.}_{4n} \), where \( X_{2n} \) is the mean fluorescence value of 2n DNA nuclei (measured in nerve cells) and S.D. is the measurement error of the system. S.D. is defined as the standard deviation of the mean value of the fluorescence values of foot mucous cells, since these cells are known to be arrested in G2 (Dübel et al. 1987). S.D. was usually about 7-7% of the 4n value.

Results

Nocodazole arrests the interstitial cell cycle at two different points

Hydras were incubated for 24 h with various concentrations of nocodazole in an attempt to arrest proliferating cells in mitosis. To detect mitotic cells, the chromatin in whole hydra was stained with the DNA-specific fluorescent dye HÖECHST 33258. Condensed chromosomes, indicating mitosis, were easily distinguished from the large round interphase nuclei of epithelial and interstitial cells and the small flattened nuclei of the nerve cells and...
nematocytes (Fig. 1A). At concentrations around 1-4 nM-nocodazole, a significant increase of cells with condensed chromosomes arranged in metaphase plates was visible in the gastric column (Fig. 1B). To demonstrate the effects of different concentrations of nocodazole on this metaphase accumulation, we counted hydra having more than 10% of their large nuclei in metaphase within an area of the body column containing about 300–400 large nuclei. We chose 10% metaphases as an amount that could not be accounted for by normal rates of proliferation. The small nuclei of non-proliferating nerve cells and nematocytes were not counted. Every hydra contained more than 10% metaphase plates after an incubation with 1-4 nM-nocodazole. The number of hydra with more than 10% metaphases decreased, however, on increasing or decreasing the concentration of nocodazole from 1-4 nM (Fig. 2). To determine the cell type responsible for the increase in metaphases, the mitotic index was calculated from cell counts of epithelial and interstitial cells in macerates of the gastric region after incubating hydra with 1-4 nM-nocodazole (Table 1). A comparison with the

mitotic index of cells from control hydra showed that only interstitial cells had been arrested in mitosis.

The large decrease in the number of animals with cells arrested in metaphase on increasing the concentration of nocodazole above 1-4 nM, as shown in Fig. 2, was surprising. The effect had not been observed in mammalian cells

![Fig. 1](image1.jpg)

**Fig. 1.** Accumulation of metaphases in the gastric column of *Hydra attenuata* after incubation with 1-4 nM-nocodazole for 44 h. A. Control, part of gastric column surface; B, after incubation with 1-4 nM-nocodazole. Nuclei and metaphase plates were visualized by fluorescent labelling of DNA in whole mounts. Arrows, metaphase plates; nn, small nuclei of nerve cells and nematocytes; in, interphase nuclei of epithelial and interstitial cells. Bar, 20 μm.

![Fig. 2](image2.jpg)

**Fig. 2.** Accumulation of metaphases at different concentrations of nocodazole. Hydra were incubated for 44 h in medium containing nocodazole and the DNA was fluorescently labelled. Gastric regions, which are known to contain the highest amount of proliferating cells, were screened for metaphases. The number of hydra are given that contained more than 10% metaphases amongst the large nuclei of epithelial and interstitial cells. The small nuclei of the non-proliferating nerve cells and nematocytes were not counted.

![Fig. 3](image3.jpg)

**Fig. 3.** Nuclear DNA contents in *Hydra* interstitial cells after incubation with 2 nM-nocodazole for 24 h. A. Nerve cells as standard for a 2n genome; B, interstitial cells of the gastric column of control hydra; C, interstitial cells of the gastric column of hydra incubated for 24 h with 2 nM-nocodazole. The arrow marked with 2n represents the mean value of the DNA content of nerve cells.

*Microtubule-dependent G2 arrest in Hydra* 349
Concentration of nocodazole (nM)

Fig. 4. Decrease of interstitial cells in the G1 or S phase of the cell cycle with increasing concentrations of nocodazole. Hydra were incubated for 24 h in nocodazole and their gastric columns were then macerated. The percentage of interphase nuclei in G1/S was determined after fluorescent labelling of DNA by quantitative microcytofluorometry of single nuclei. Cells were interpreted to be in the G1 or S phase of the cell cycle when their nuclear fluorescence value was significantly smaller than 4n. The threshold was defined as twice the mean fluorescence value of nerve cells minus the standard deviation of cells known to be arrested in G2.

Fig. 5. Decrease of interstitial cells in G1/S with time after the addition of 2 nM-nocodazole. The percentage of cells in G1/S was determined as described in Fig. 4.

Fig. 6. Synchronization of interstitial cell cycles with 2 nM-nocodazole. Hydra were incubated for 24 h in nocodazole, washed and transferred to nocodazole-free medium. The percentage of interphase cells in G1/S was determined at different time points starting with the washing step as described in Fig. 4 (••). (O) Control.

Fig. 7. Reaction of tubulin monoclonal antibodies with the Western blot of a Hydra lysate. Hydra were sonicated in electrophoresis sample buffer and applied to a SDS–12 % polyacrylamide gel. The proteins were blotted onto nitrocellulose and tubulin was identified using tubulin monoclonal antibodies and an alkaline phosphatase-conjugated second antibody. Lane a, control, purified pig brain tubulin detected with DM1A; lanes b,c,d, Hydra lysate incubated with DM1A, DM1B and YOL1/34, respectively.
Fig. 8. Microtubule immunofluorescence in single cells of macerated hydra. A. Ectodermal epithelial cell, basal part on the left (arrow: part of an endodermal epithelial cell); B, nerve cell; C, cluster of four interstitial cells; D, nematocyte and its cnidocil; E, epithelial cell in mitosis. Left, phase-contrast; right, immunofluorescence. Bar, 20 μm.

dence of this G₂ arrest, the nuclear DNA content of interphase interstitial cells from hydra incubated for 24 h in various concentrations of nocodazole was determined. Interstitial cells with a DNA content significantly lower than 4n represent cells in the very short, if at all existent, G₁ phase or the S phase of the cell cycle (Campbell & David, 1974) and are referred as 'cells in G₁/S'. The proportion of interphase interstitial cells in G₁/S was observed to decrease after incubating hydra with concentrations of nocodazole above 1 nM (Fig. 4). At concentrations above 2 nM-nocodazole, all of the interphase interstitial cells were arrested in G₂.

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Table 1. Arrest of Hydra interstitial cells in metaphase after incubation of 1-4 nM-nocodazole

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Treatment with nocodazole</th>
<th>Mitotic index ± s.d. (%)</th>
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<tbody>
<tr>
<td>Interstitial cells</td>
<td>-</td>
<td>3.4 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>3.0 ± 1.8</td>
</tr>
<tr>
<td>Epithelial cells</td>
<td>-</td>
<td>0.3 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>0.0 ± 0.0</td>
</tr>
</tbody>
</table>

After incubating hydra for 44 h in culture medium containing nocodazole, the gastric parts were cut out and macerated. S.D., standard deviation in three experiments. About 500 cells of each type were counted in each experiment.

Microtubule immunofluorescence

Nocodazole and other microtubule inhibitors are usually used at much higher concentrations than 2 nM to arrest cell cycles in mitosis. To determine, therefore, whether such low concentrations of nocodazole were indeed affecting hydra's microtubules, they were visualized using the monoclonal antibody DM1A. This anti-tubulin monoclonal antibody was selected from three commercially available tubulin monoclonal antibodies after testing their reaction against a Western blot of a Hydra lysate. DM1A reacted strongly with a band of the same molecular weight as pig brain tubulin and showed no cross-reactions with other proteins (Fig. 7). The microtubule distributions in hydra were demonstrated by incubating cells or tissue with DM1A and a second fluorescent antibody. The specificity of DM1A for microtubules was checked using the monoclonal antibodies DM1B and YOL1/34, whose epitopes are known to be different from that of DM1A (Blosee/1, 1984; Breitling & Little, 1986). They both stained the same structures as DM1A. Furthermore, no immunofluorescent structures were visible after preincubation of DM1A with 20 μM pig brain tubulin.

Mitotic spindles, the cytoplasm of ectodermal epithelial cells, and the neuritic extensions of nerve cells were strongly fluorescent (Fig. 8A, B, E). In interstitial cells, the brightest immunofluorescence was seen in bridges connecting the typical 2° cell clusters (Fig. 8C). Nematocytes showed highly fluorescent structures in the cnidocil and in the conical part of the cell (Fig. 8D), in agreement with the distribution of microtubules as observed by electron microscopy (Slatterback, 1963, 1967). The microtubule structures of single cells were also recognized in whole mounts and thin sections of hydra.
Fig. 10. Microtubule immunofluorescence in tentacles of *H. attenuata*. A. Nerve pathways along tentacles connecting battery cell complexes; B, six battery cell complexes in a partially contracted tentacle; C, part of a relaxed tentacle; D, proximal part of a tentacle. A neuritic network connecting nematocytes and nerve cells can be seen in both C and D. Arrows, nematocytes of different size; arrowheads, cnidocilia; np, nerve cell perikarya. Bars: A, 100 μm; B, 20 μm.

Hydra. Fig. 9 shows a whole mount of a hydra and a view of the body column. The basket-like arrangement of microtubules in nematocytes when viewed from the side as in Fig. 8D appeared as a ring when viewed from above. Mitotic spindles could also be observed in the body column of hydra as shown in Fig. 9B. Other microtubule structures, however, were difficult to identify due to the intense immunofluorescence of microtubules in epithelial cells. In contrast, terminally differentiated epithelial cells of tentacles showed no microtubule immunofluorescent structures (Fig. 10). It was therefore possible to observe the fluorescent microtubule structures of other tentacle cells more clearly. These included the intensely fluorescent basket-like structure surrounding the nematocyte, the small bristles radiating from the nematocytes representing the sensory cnidocils and the thread-like connections between nematocytes of the same and different battery cell complexes. From comparisons with ultrastructural and immunological investigations (Grimmelikhuijzen, 1983; Westfall & Kinnamon, 1984; Yu et al. 1985; Yaross et al. 1986) these threadlike connections were identified as neurites. Microtubule immunofluor-
Microtubule immunofluorescence was largely absent from the endoderm as shown in thin sections (Fig. 11). The more intense fluorescence of the tentacle ectoderm in sections was due to its relatively high concentration of nematocytes.

Differential microtubule disassembly using low concentrations of nocodazole

To investigate the effect of nocodazole on Hydra microtubules, microtubule immunofluorescence in thin sections and whole mounts of animals was examined after incubation with various concentrations of nocodazole for 24 h (Fig. 12). After incubation of hydra with 3-4 nM-nocodazole, the microtubule immunofluorescence typical of nerves and of the interstitial and epithelial cells in the body wall was no longer visible. The cnidocil microtubules appeared to be more resistant. They were still visible, although reduced in number, after incubation with 10 nM-nocodazole. Nematocytes appeared to contain the most stable microtubule structures. After incubation with 34 nM-nocodazole, their typical microtubule structures were beginning to disintegrate but were still visible in some cells. Cnidocil microtubule immunofluorescence had completely disappeared. After incubation with 100 nM-nocodazole, the typical basket-like microtubule immunofluorescence around nematocytes was destroyed and dispersed in the cytoplasm, surrounding the nematocyte nucleus. A summary of these observations is shown in Table 2.

Having found that some Hydra microtubule structures were indeed affected by very low concentrations of nocodazole, we made a more detailed investigation of the effect of 2 nM-nocodazole, the concentration used to synchronize interstitial cells. The results are shown in Fig. 13. The microtubule immunofluorescence of the body of hydra was largely accounted for by nematocytes; immunofluorescence from interstitial and epithelial cells had disappeared (cf. Fig. 9). Nerve cell microtubules had also largely disappeared from the body column, but they were still visible in the extremities. The neuritic network of the peduncle, for example, was still fairly intact in

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Fig. 11. Microtubule immunofluorescence in thin sections of *H. attenuata*. A. Longitudinal section through a whole animal demonstrating the ectodermal location of most microtubules; B, section through a foot; C, phase-contrast of B. *ec*, Ectoderm; *en*, endoderm; *mu*, foot-specific adhesion mucus. Bars: A, 200 μm; B, 20 μm.

Fig. 12. Effect of nocodazole on the microtubules of different *Hydra* cell types. Hydra were incubated with various concentrations of nocodazole for 24 h and the microtubules labelled by immunofluorescence. A. 0-34 nM-nocodazole; a thin section of the body wall showing microtubule immunofluorescence in the whole ectoderm and in endodermal nerve cells. B. Phase-contrast of A; C-F, 3-4 nM-nocodazole; thin sections of the body wall showing that the microtubules of nematocytes and cnidocils (arrowheads) were still present, whereas they had disappeared from interstitial and epithelial cells. D,F. Phase-contrast of C,E, respectively. G, 10 nM-nocodazole; a tentacle showing microtubule immunofluorescence only in nematocytes and a few cnidocils (arrowheads). H, 34 nM-nocodazole; a tentacle stump showing the start of microtubule disintegration in nematocytes; endocilia were no longer visible. I. 100 nM-nocodazole; the typical ‘basket’ structure of microtubules in nematocytes were destroyed; microtubule immunofluorescence can be seen dispersed in the cytoplasm surrounding the nucleus (arrow). *ec*, Ectoderm; *en*, endoderm; *m*, mesogloea; *n*, nematocyte; *ne*, nerve cells; *s*, mitotic spindle. Bars: A-F and I, 20 μm; G,H, 100 μm.
Microtubule-dependent $G_2$ arrest in Hydra
Fig. 13. Microtubule immunofluorescence in *H. attenuata* incubated with 2 nm-nocodazole for 24 h. A. Whole hydra; microtubule immunofluorescence of nematocytes after the disappearance of microtubules from epithelial and interstitial cells (cf. Fig. 9). B. Neuritic network in the peduncle; arrows, nerve cell perikarya. C. Hypostome demonstrating sensory nerve cells (arrows); above, a tentacle is visible showing microtubule immunofluorescence in nerve cells, nematocytes and cnidocilia; a lack of neurite-like microtubule immunofluorescence in the basal part of the hypostome (*h*) contrasts with that of intact nerve processes in the tentacle. D. Body wall of the gastric column demonstrating the absence of mitotic spindles and nerve cell microtubules. E. The same area as D stained for DNA to demonstrate condensed metaphase chromosomes (arrow). *h*, Hypostome; *te*, tentacle; *n*, nematocytes. Bars: A, 500 μm; B,D, 20 μm; C, 100 μm.
Animals incubated with up to 8 nM-nocodazole, but not 10 nM-nocodazole, were able to resume feeding after several days of reconvalescence and grow. To investigate the effect of nocodazole concentrations known to destroy neurite microtubules on the feeding response, animals were incubated with various concentrations of nocodazole for 24 h and then assayed for feeding reactions by adding shrimps or shrimp extract. The moderately contracted tentacles of hydra treated with 2 nM-nocodazole were still able to respond to feeding; nematocyte discharge, tentacle movements and mouth movements still occurred (Table 3). However, whereas tentacular movements ceased completely after incubating hydra with 4 nM-nocodazole, mouth movements and nematocyte discharge were not affected. It appears, therefore, that intact microtubule structures in neurites are not required for mouth movements and nematocyte discharge.

Discussion

Microtubule structures in hydra – effect of nocodazole

It is interesting to note that the term 'microtubules' was used for the first time 25 years ago by Slautterback (1963) in his description of these organelles in the interstitial cells and nematocytes of hydra. In agreement with his ultrastructural investigations (Slautterback, 1963, 1967) our immunofluorescence procedure using a tubulin monoclonal antibody showed a particularly high concentration of microtubules in the conical part of the drop-shaped nematocyte. We were also able to observe mitotic spindles and the microtubule distribution in single cells, thin sections and whole mounts.

Furthermore, the neuritic network connecting nematocytes and neurones in Hydra tentacles was clearly visible due to the absence of microtubule immunofluorescence in the epithelial cells. Similar neuritic connections have also been observed using a monoclonal antibody to a protein found mainly in neurones (Yu et al. 1985). The absence of microtubules in tentacular epithelial cells is correlated...
Table 2. Sensitivity of Hydra microtubules to nocodazole

<table>
<thead>
<tr>
<th>Nocodazole concentration (nM)</th>
<th>Cytoplasm</th>
<th>Spindles</th>
<th>Nerves</th>
<th>Cnidocils</th>
<th>Nematocytes</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>340 µM</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>1 nM</td>
<td>±</td>
<td>±</td>
<td>±</td>
<td>±</td>
<td>±</td>
</tr>
<tr>
<td>3.4 nM</td>
<td>-</td>
<td>-</td>
<td>±</td>
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<td>10 nM</td>
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<tr>
<td>24 nM</td>
<td>-</td>
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<tr>
<td>100 nM</td>
<td>-</td>
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</table>

Hydra were incubated in medium containing nocodazole for 24 h prior to antibody staining in whole mounts and thin sections. +, presence of the typical immunofluorescent structures seen in whole mounts and tissue sections (cf. Figs 9, 10, 11); ±, presence of these structures in part of the investigated material; −, lack of the typical immunofluorescent structures (cf. Figs 12, 13).

Table 3. Effect of different concentrations of nocodazole on feeding behaviour in Hydra

<table>
<thead>
<tr>
<th>Nocodazole (nM)</th>
<th>Tentacle movement</th>
<th>Mouth opening</th>
<th>Nematocyte discharge</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2-0</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>4-0</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>6-0</td>
<td>-</td>
<td>(+)</td>
<td>+</td>
</tr>
<tr>
<td>10-0</td>
<td>-</td>
<td>(+)/—</td>
<td>+</td>
</tr>
</tbody>
</table>

Hydra were incubated for 24 h in medium containing nocodazole. After washing in medium, the feeding reaction was induced by adding crude shrimp extract (10 000 g supernatant of sonicated A. salina) and controlled with a binocular microscope. +, Normal reaction (within 60 s after induction); (+), all hydra reacted, but weaker response (decreased opening diameter); (+)/—, only a few hydra reacted. Nematocyte discharge was tested by adding Artemia and defined as attachment of the shrimps such that they cannot be easily blown away with a beam of medium.

with a stage at which these cells are terminally arrested in the G2 phase of the cell cycle (Dübel et al. 1987). This lack of tubulin expression may therefore be useful as a differentiation maker for ectodermal epithelial cells of the tentacles. A simplified diagram of tentacular and body wall sections that emphasize the distribution of microtubules is shown in Fig. 15.

Differences in the sensitivity of Hydra microtubules to nocodazole could be due to associated proteins, post-translational modifications or function-specific tubulins. The number of different tubulin isotypes in Hydra is unknown, and although sequence data on over 60 tubulin subunits are now available (for review, see Little & Seehaus, 1988), tubulins from the evolutionary important Phyla of coelenterates have not been analysed. It is known, however, that Hydra tubulins, unlike other tubulins, do not appear to bind colchicine with high affinity. Microtubules were still visible in Hydra cells after incubation with 10 µM-colochicine (Campbell, 1976). In contrast, it is clear from our present work that one or more Hydra tubulins have an extremely high affinity for nocodazole. Spindles and stem cell microtubules, for example, disappeared at only 0-8-1 nM-nocodazole. These concentrations of nocodazole are much lower than those required for the disruption of mammalian microtubules. For example, 130 nM-nocodazole is required for the efficient arrest of mitosis in several mammalian cell lines (DeBrabander et al. 1976; Zieve et al. 1980) and 7 µM-nocodazole is required to inhibit mammalian brain microtubule assembly by 50% (Kilmartin, 1981). Similar concentrations of nocodazole are also necessary for the disruption of yeast microtubules (Kilmartin, 1981).

Cell cycle arrest in G2

Significant amounts of hydra interstitial cells were arrested in metaphase after incubation with 1 nM-nocodazole. Interphase cells in G1/S and G2 were present in approximately equal amounts. After incubation with 2 nM-nocodazole, however, only a minor proportion of the interstitial cells were in metaphase and all of the interphase interstitial cells had been arrested in G2. At these extremely low concentrations of nocodazole, the observed effects can only be due to very specific reactions. The observed correlation of cell cycle arrest with microtubule disassembly of spindle and cytoplasmic microtubules strongly indicates that nocodazole is reacting specifically with tubulin. In mammalian cells, the lack of G2 arrest may possibly be due to the much lower sensitivity of their microtubules to nocodazole.

Removal of the 2 nM-nocodazole block led to a wave of mitoses after 7-8 h followed about 2 h later by the entry of 75% of the interphase interstitial cells in G2/S of the next cell cycle. The synchronous passage of interstitial cells into G2/S indicates that most of them were arrested at a similar point in G2. The length of G2 in interstitial cells has been shown to vary between 4 and 22 h, and mitosis lasts about 1 h (Campbell & David, 1974). It appears, therefore, that the lapse of time between the G2 arrest point and mitosis is much less variable than the length of G2 itself.

An extra small wave of cells in G2/S preceding the major peak was not observed, indicating that cells arrested in metaphase had not been able to recover. This is in agreement with the finding that mammalian cells arrested for longer periods of time in metaphase with nocodazole lose their ability to return to interphase (Zieve et al. 1980). It is probably fortuitous, therefore, that nocodazole blocks most of the interstitial cell cycles in G2, since they can be held in this phase for relatively long periods without adverse effects. To our knowledge
this is the first report of cell cycle synchronization by G₂ arrest.

The steps following G₂ arrest and leading to mitosis seem to be dependent on the assembly of a microtubule-dependent structure. Without it the series of events leading to nuclear membrane breakdown and chromosome condensation are blocked. A possible candidate might resemble the novel dot-like structure recently identified by tubulin immunofluorescence that appeared 60 min before metaphase in nuclei of the slime mould Physarum polycephalum (Paul et al. 1987). The assembly of this or another microtubule structure involved in Physarum cell cycle regulation might start even earlier, seem to be dependent on the assembly of a microtubule before mitosis delayed chromosome condensation and this is the first report of cell cycle synchronization by G₂ dependent structure. Without it the series of events of other primitive organisms. The synchronization of rapidly dividing stem cells in Hydra and possibly other organisms should also provide a useful starting point for answering questions concerning their differentiation.

Unlike colchicine, nocodazole has been shown to have a high affinity for the microtubules of taxonomically distant species. The use of this mitotic inhibitor and tubulin antibodies might therefore be equally useful for studying microtubule-dependent functions of other primitive organisms. The synchronization of rapidly dividing stem cells in Hydra and possibly other organisms should also provide a useful starting point for answering questions concerning their differentiation. However, perhaps the most intriguing questions raised by our results are what is the nature of the putative microtubule structure in the G₂ phase and how does it regulate the progress of the cell cycle towards mitosis?

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