The Glu-Modification of α-Tubulin in the Feeding Apparatus of the Primitive Flagellate *Entosiphon sulcatum* Is Only Apparent after Detergent Treatment

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Using specific monoclonal antibodies, we investigated the distribution of post-translational modified Tyr- and Glu-tubulins during interphase of the primitive flagellate *Entosiphon sulcatum*. Immunofluorescence studies of simultaneously permeabilized and fixed cells revealed that microtubular structures comprising $Ca^{2\oplus}$-labile subpellicular and flagellar MTs and $Ca^{2\oplus}$-stable MTs in the siphon complex (feeding organelle) reacted surprisingly unorthodox with antibodies against Tyr- and Glu-tubulin: Unexpectedly, the siphon complex consisting of $Ca^{2\oplus}$-stable MTs appeared exclusively Tyr-positive, whereas the $Ca^{2\oplus}$-labile subpellicular and flagellar MTs reacted with the Glu- as well as with the Tyr-antibody. That the siphon MTs were indeed $Ca^{2\oplus}$-stable and all other MTs had become solubilized, was verified by EM-observation.

This surprising result contrasting considerably with the permanent nature of the siphon complex, was reconsidered after preceding lysis and extraction procedures. Depending on the type of detergent used and on extraction times applied, the MTs of the siphon complex now always showed also Glu-positivity, indicating the presence of detyrosinated α-tubulin as a biochemical marker of stabilized MTs. Since saponin, irrespective of subsequent extraction times, always produced a Glu-positive reaction and ultrastructural analysis never gave compelling evidence for a drastic MAP-removal, we conclude that the Glu-epitope became freely accessible due to conformational changes in the tubulin polymeres.

Introduction

In recent years, the species *Entosiphon sulcatum*, a representative of the heterotrophic flagellates belonging to the order Euglenida, was due to its complex feeding apparatus repeatedly subject of elaborate ultrastructural studies (Mignot, 1963; Triemer and Fritz, 1987; Nahrebne and Triemer, 1990; Belhadri and Brugerolle, 1992; Belhadri *et al.*, 1992). These protists posess a cytoskeleton composed mainly of the microtubular feeding organelle (siphon), subpellicular microtubules (MTs) and the recurrent and anterior flagella.

The siphon complex is a true axial cytoskeleton composed of three microtubular rods arranged in a "C-shaped" configuration with a dorsal opening. Each rod consists of a large number of interconnected MTs arranged in parallel array running from the anterior to the posterior region of the cell. It is supposed that this peculiar organelle is in some way engaged in bacterial food uptake (Mignot, 1966), but at present there is no clear proof for the exact way of food uptake.

At the onset of division, the parental organelle – in contrast to the cytopharyngeal structures of ciliates (Hausmann and Peck, 1979) – depolymerizes completely and new microtubular arrays develop in the daughter cells.

In the course of our studies on the development of the new organelle, the characterization of tubulin post-translational modifications seemed to be especially informative, since MT-containing structures in protists often exhibit dramatic changes related to their life cycle (Sherwin *et al.*,

Abbreviations: CLSM, confocal laser scanning microscopy; DTAF, 5-([4,6-dichlorotriazin-2-yl]amino)fluorescein; EGTA, ethylene-glycol-bis(b-aminoethyl-ether)-N,N,N',N'-tetraacetic acid; EM, electron microscopy; HEPES, N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid); IIF, indirect immunofluorescence; MAP, microtubule-associated protein; MTs, microtubules; PBS, phosphate buffered saline; PIPES, piperazine-N,N'-bis(2-ethanesulfonic acid); TCP, tyrosine carboxypeptidase; TEM, transmission electron microscopy.

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1987; Delgado-Viscogliosi *et al.*, 1996). In the case of trypanosomes all microtubule arrays in the cell contain some Tyr-tubulin, and it appears that no isotype in the trypanosomes is excluded from tyrosination, but with time a significant decrease in tyrosinated tubulin is noticeable (Sherwin *et al.*, 1987). This suggests a correlation between persistence of MTs through time and an increase in their post-translational loss of tyrosine and indeed in all long-lived microtubular organelles an increasing detyrosination by the action of a tyrosine carboxypeptidase (TCP) was regularly detected (for review see Bulinski and Gundersen, 1991).

To our surprise, the feeding apparatus of *Entosiphon*, however, revealed completely Tyr-positive throughout the whole life cycle and showed always Glu-negativity when processed by conventional methods with appropriate monoclonal antibodies for immunofluorescence (IIF).

Material and Methods

Entosiphon sulcatum was isolated from raw cultures of *Reticulomyxa filosa* originally collected from ponds in the green houses of the Botanical Gardens at the Ruhr-University Bochum. Cells were grown in Petri dishes (∅ 9 cm) with mineral water (Volvic) on wheat grains for several weeks to high density.

For IIF cells were either fixed with 4% (w/v) formaldehyde (freshly prepared from paraformaldehyde) in PBS containing 1% (v/v) Triton X-100 respectively saponin or after preceding lysis with 0.15% (w/v) Brij 58 respectively saponin in 50% PHEM (60 mM PIPES; 25 mM HEPES; 8 mM EGTA; 2 mM $MgCl_2$; pH 7) containing 5% (v/v) hexylene glycol for 2 minutes (Koonce and Schliwa, 1986). For observation, cells were attached onto alcian blue (0.1% (w/v) alcian blue 8 GX in 3% acetic acid/ethanol) coated cover slips. Subsequently, lysed cells were washed 3 times for 1 min in PEM-buffer (100 mM PIPES; 2 mM EGTA; 1 mM $MgSO_4$; pH 6,9) (Simon and Salmon, 1990). The lysed cells were after the PEM washing either processed directly for IIF or after a 5 min respectively 10 min extraction period with or without 6 mM $CaCl_2$. Following a short PBS rinsing, cover slips were loaded with the primary antibody YL 1/2 against Tyr-tubulin or with the antibody ID5 against Glu-tubulin (both monoclonal antibodies were a generous gift of Dr. J. Wehland, GFB/Braunschweig) for 1 h in a moist chamber at 37 °C. After that, cells were routinely washed 3 times in PBS (3 x 5 min) and immersed with the secondary antibodies (IgG-DTAF from Dianova/Hamburg) anti-mouse respectively anti-rat (diluted 1:20) for another hour, then repeatedly rinsed in PBS and finally embedded in anti-fading medium (87% glycerol/PBS with 0.1 g p-phenylendiamine).

Slides were observed with a Zeiss-Photomikroskop II equipped with epifluorescence condenser III RS and planapochromate 63 x (1.4) or 40 x (1.0) objectives. For the documentation of fluorescence intensities a MRC-600 Laser Scanning Confocal Imaging System (CLSM) from BIO-RAD mounted on a Zeiss Axiovert 35 was also used. The optical section levels were summed up and processed with BIO-RAD software. Photographs were either taken on Kodak T-Max 400 or on Kodak T-Max 100 respectively on Fujichrome 100 film.

For transmission electron microscopy (TEM), cells were pelleted for 5 min at 3,000 rpm and fixed for 30 min in 2.5% (v/v) glutaraldehyde/0.05 M Na-cacodylate (pH 7) and after rinsing in the buffer (15 min) post-fixed for another 30 min in 1% (w/v) OsO_4/0.05 M Na-cacodylate. After washing with the buffer, cells were dehydrated in ethanol and embedded in Epon 812. After double staining as usual, sections were examined with a Philips EM 410 at 80 kV.

Lysed cells were processed for TEM with a modified procedure according to Hohenberg *et al.* (1994) in cellulose capillary tubes (kind gift of Dr. Hohenberg, Hamburg): pelleted cells were sucked into the tubes (200 µm ∅) by capillary forces and transferred into the lysis medium for 2 min and after incubation in washing buffer (3 x 1 min) extracted with the PEM-buffer for different time intervals as described above. Finally, the tubes were plunged into the fixation medium (see above) and treated as usual.

Results and Discussion

Although the ultrastructure of the feeding apparatus (siphon) of *Entosiphon* has already been subject of several studies (Mignot, 1963; Mignot, 1966; Triemer and Fritz, 1987; Belhadri and Brug-

erolle, 1992), the complexity of its organization needs a brief redescription. As seen in the diagram of the cross-sectioned anterior quarter of the siphon region (Fig. 1A) as well as in the corresponding micrograph, three separate microtubular bundles form a rim or furrow (Fig. 2) since a single row of MTs encircles the three rods at their utmost end almost completey. An amorphous electrondense substance, which Belhadri *et al.* (1992) describe as cement material, incrusts in the anterior region the microtubular structures externally (Fig. 1A, C and Fig. 2). Towards the posterior end, the number of interlinked parallel MT-rows gradually decrease and also the cement material disappears in the more posterior part of the siphon (Fig. 1B, C). The luminal region of the furrow in the median part of the siphon (Fig. 1A, C and Fig. 2) contains sigmoid curved vane-like structures originating from the margin of the posterior rod structures. These vanes consist of an electrondense filamenteous sheet linked to a centrally located single MT (Fig. 2, v) terminating anteriorly at the peripheral MT-rows of the siphon structure.

When fixed and simultaneously permeabilized cells (Fig. 3a, b) were labelled by the monoclonal antibody YL 1/2 against tyrosinated tubulin, except the anterior quarter, the siphon complex exhibited a brillant Tyr-positive reaction (Fig. 3d). Remarkably, all other cytoskeletal components, excluding the flagella and basal bodies, showed no or at least a very weak reaction. In contrast to this, Glu-antibodies indicating the detyrosinated form of α-tubulin, revealed a complete negative reaction of the siphon complex, whereas the subpellicular MTs and also the flagella showed clear positivity (Fig. 3c). Additionally, also the nucleus gave a strong positive reaction.

Implicit in the concept of the permanent cytoskeletal elements is the relative stability of the MT-systems that comprise it. It came as a surprise, therefore that the MT-populations of the siphon, due to their exclusive Tyr-stainability, appear as a rapidly turning over subclass (for references see Barra *et al.*, 1988), exchanging subunits with the soluble pool with half-times reported for highly dynamic MTs (Webster *et al.*, 1987; Wehland and Weber, 1987; Greer and Rosenbaum, 1989). Apparent diversities regarding stability among MTs in many different MT-systems have repeatedly been reported (Bulinski *et al.*, 1988; Warn *et al.*, 1990). Such diversities usually reflect different posttranslationally modified tubulins (Barra *et al.*, 1988; Bulinski and Gundersen, 1991), which, as a consequence, attain different binding capacities for stabilizing MT-associated proteins (MAPs). It is widely accepted that more stable MT-structures are enriched in the Glu-modification of α-tubulin (e.g. Webster *et al.*, 1987; Greer and Rosenbaum, 1989), whereas Tyr-α-tubulin represents the more labile MT-subclass (Wehland and Weber, 1987).

That the MTs comprising the siphon complex, in spite of their supposed Glu-negativity, indeed represent a stable subclass was tested by the incubation of Brij 58-lysed cells with PEM-buffer containing 6 mM $Ca^{2\oplus}$. The extreme sensitivity of native MTs against millimolar $Ca^{2\oplus}$-concentrations is well known, e.g. Kiehart (1981). Stolz and Bereiter-Hahn (1988) demonstrated a gradual loss of MTs in XTH-2 cells even after exposure to 1 μM $Ca^{2\oplus}$ with increasing incubation time.

Our own electron microscopic results clearly suggest that in *E. sulcatum* at least two classes of different MT-stabilities exist: a $Ca^{2\oplus}$-sensitive fraction of subpellicular and flagellar MTs and a $Ca^{2\oplus}$-insensitive class of siphon-MTs. At the ultrastructural level, after a prolonged $Ca^{2\oplus}$-incubation (10 min), only a few MTs in the marginal area of the siphon complex, as evidenced by their v- or c-shape, appear somewhat disintegrated (arrowhead in Fig. 4a, insert). Merely, the cement material normally incrusting the peripheral MT-rows, has become almost solubilized (Fig. 4a). Furthermore, the crossbridges spacing the distance between the single MT-rows appear in most cases more or less impaired, whereas the shorter intra-row bridges are quite intact (Fig. 4a and insert).

Since the YL 1/2 antibody against tyrosinated α-tubulin hardly stained the anterior quarter of the siphon, we again tested the Tyr-labelling after the lysis procedure, when the marginal cement material has been vastly solubilized. Obviously, the cement material, which usually coats the upper part of the siphon, indeed obstructs the antibody reaction, since after the removal of the cement, Tyr-positivity was present over the total length of the siphon in an overall high intensity.

It seemed not unlikely that due to the cement masking, the anterior siphon region likewise did not react with the Glu-antibody, therefore, we also tested the reaction of the ID5 antibody after Brij

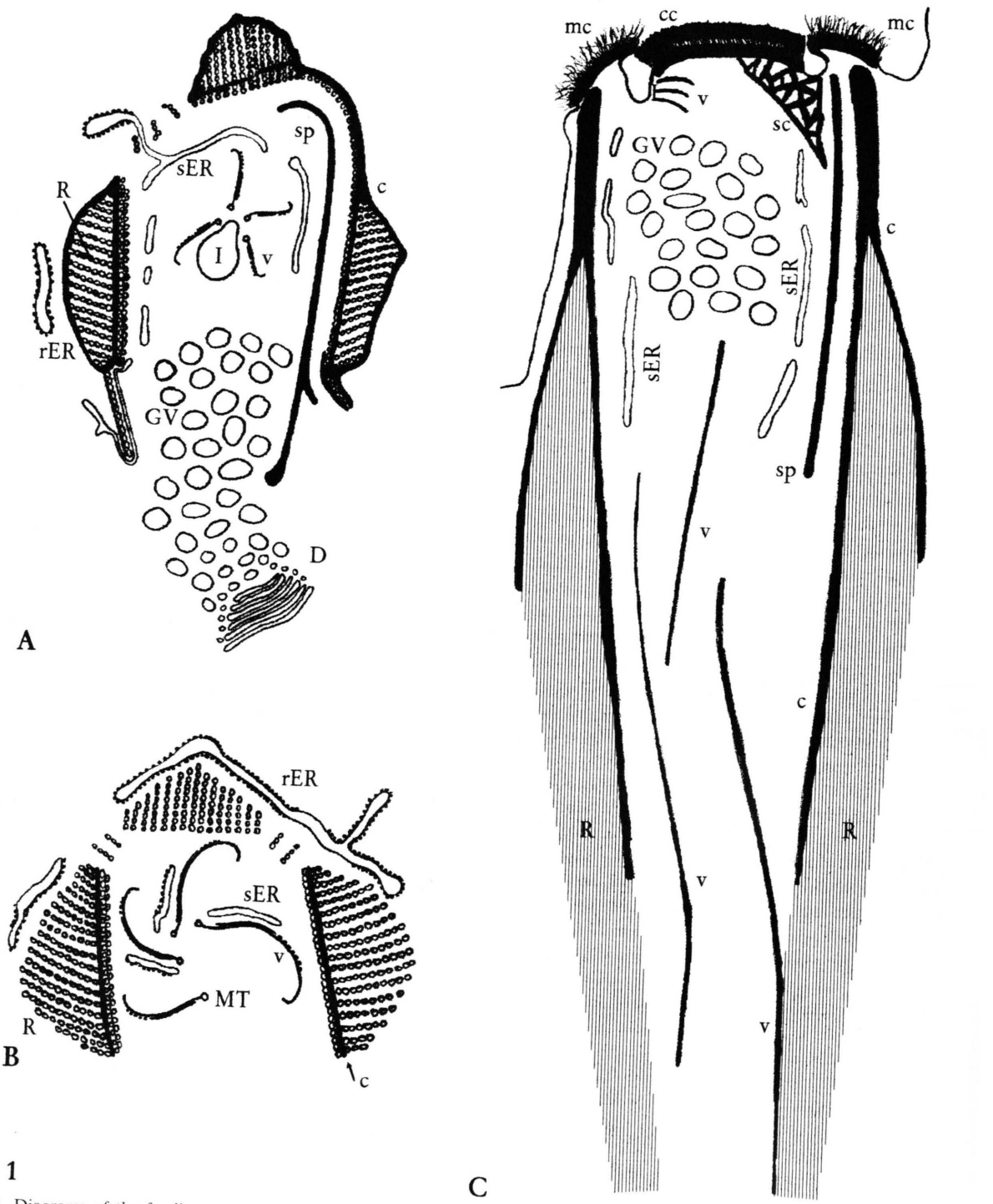

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Fig. 1. Diagrams of the feeding apparatus of *Entosiphon sulcatum*. A: Cross-section of the anterior part; B: Cross-section of the median part; C: Longitudinal-section.
Abbreviations: c, cement; cc, central cap; D, dictyosom; GV, Golgi-vesicle; I, invagination of plasmamembrane; mc, microtubular cap; R, microtubular rod; rER, rough endoplasmic reticulum; sc, scaffold; sER, smooth endoplasmic reticulum; sp, supplementary plaque; v, vane.

Fig. 2. EM-micrograph of the cross-sectioned anterior siphon region: The feeding apparatus (siphon) consists of three "C-shaped" arranged microtubular rods (R). The MTs within the rods appear in linear arrays connected by short intra-row (arrowheads) and longer inter-row bridges (arrows). Four vanes (v) are located centrally in the lumen of the siphon. In the anterior part, the microtubular organelle is peripherally embedded in an electron-dense cement-like material (c).
ER, endoplasmic reticulum; F1, anterior flagellum; F2, recurrent flagellum; GV, Golgi vesicle; sp, supplementary plaque. Bar: 0.5 μm.

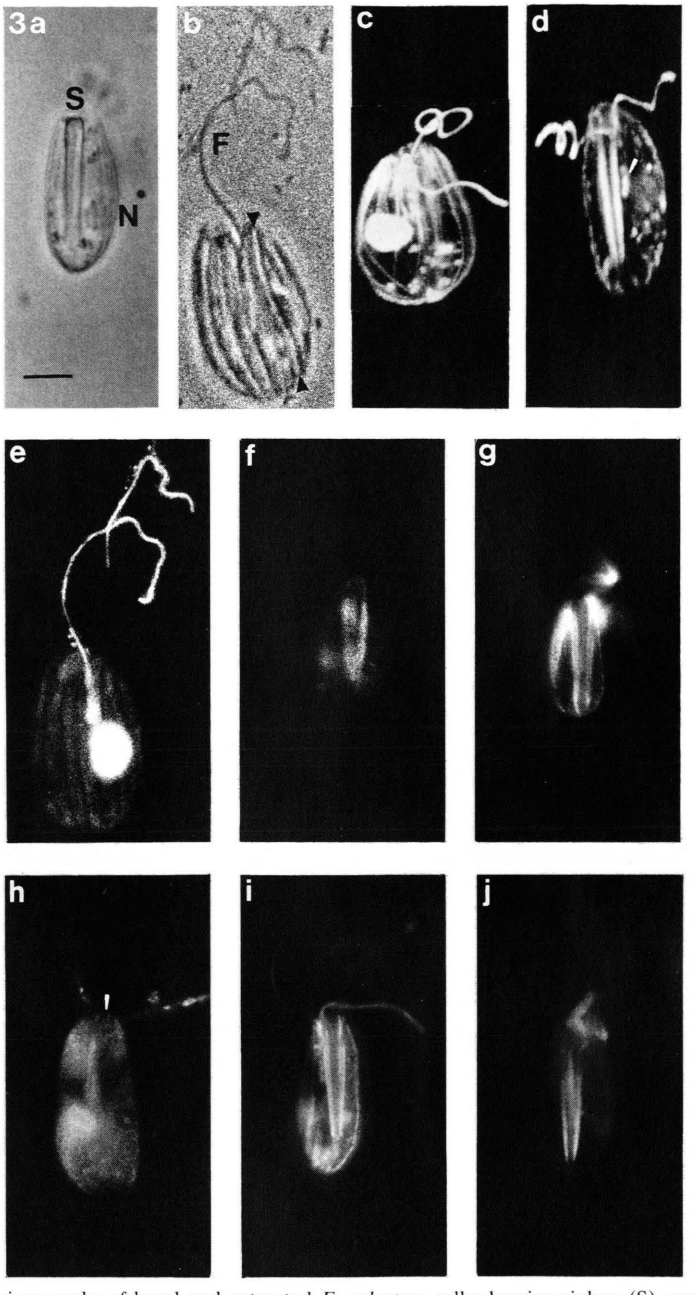

Fig. 3. a,b: Phase contrast micrographs of lysed and extracted *E. sulcatum* cells showing siphon (S), nucleus (N), flagella (F), and pellicle-grooves (arrowheads). Bar: 5 μm. c-j: Immunofluorescence micrographs of Tyr- and Glu-tubulin-antibody labelled cells after various lysis and extraction procedures. c: CLSM-micrograph of Glu-antibody labelled *E. sulcatum* after simultaneous permeabilization and fixation. Note absence of siphon staining and the Glu-positive reaction of flagella and subpellicular MTs. d: CLSM-imaged Tyr-antibody labelled cell revealing the Tyr-positive siphon complex together with the flagella. The arrowhead points to the basal body complex. e: Glu-antibody (ID5) reaction after Brij 58-lysis without subsequent PEM-extraction. Only the flagella and subpellicular MTs appear Glu-positive. Note also the staining of the nucleus. f: After Brij 58-lysis and a 5 min PEM-extraction with 6 mM $Ca^{2\oplus}$, the siphon stains slightly Glu-positive. Note the disintegration of all other microtubular structures. g: After Brij 58-lysis and a 10 min PEM-extraction (without $Ca^{2\oplus}$), the siphon complex stains brightly with the Glu-antibody (ID5). h: Saponin permeabilized and simultaneously fixed cell with Glu-negative reaction of the siphon complex (arrowhead). Since the focus was positioned on the siphon, the subpellicular MTs and the flagella produce a halo. i: Short saponin lysis preceding fixation leads to a brillant Glu-positive reaction of all microtubular structures including the siphon. j: Tyr-positive reaction of the siphon complex. Fixation and lysis conditions as in Fig. 3i.

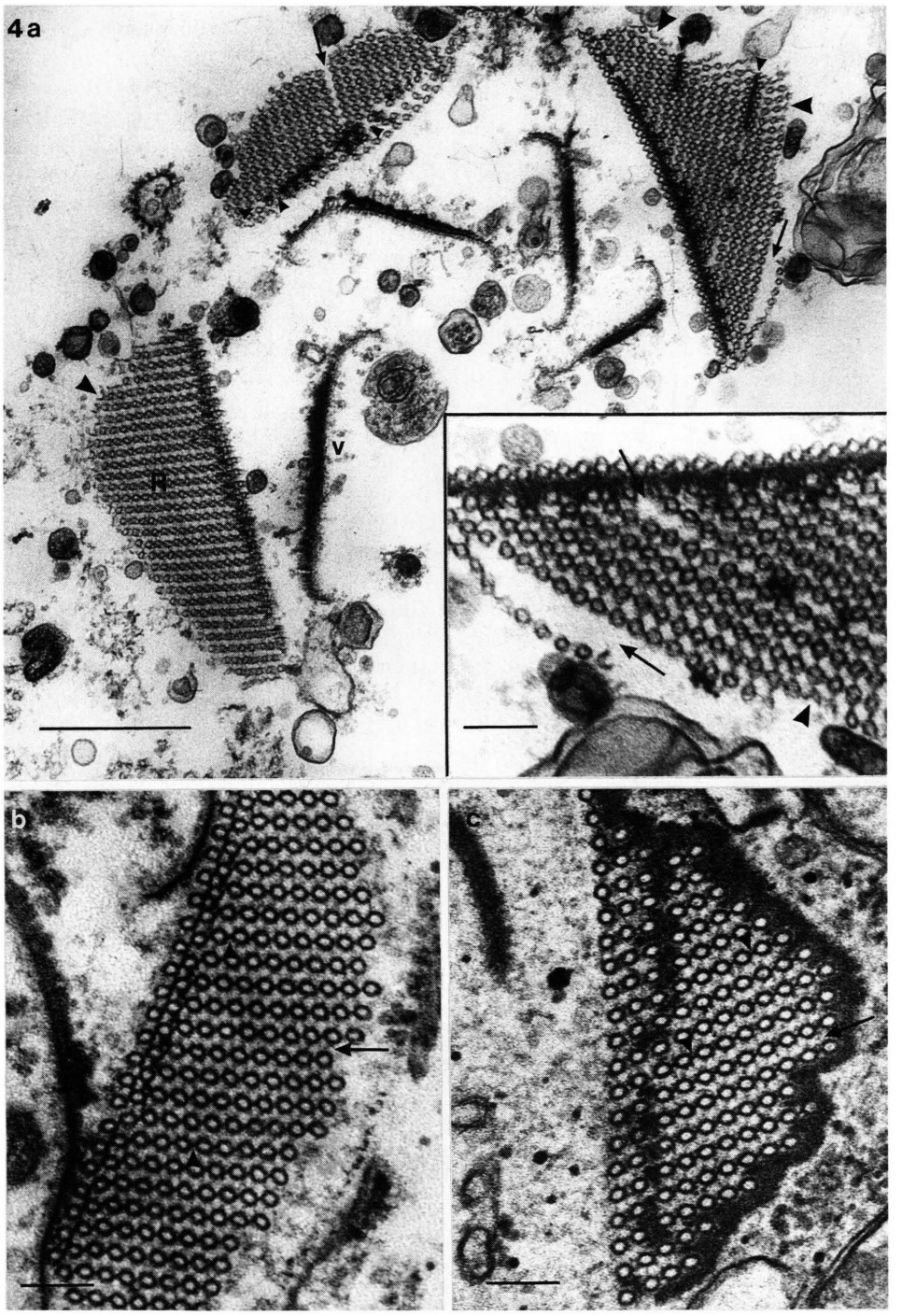

Fig. 4. 4a with insert: Cross-sectioned median siphon region after Brij 58-lysis and a 10 min extration with 6 mM $Ca^{2\oplus}$-containing PEM-buffer. Note the partial disintegration of the cement-like material (small arrowheads) and of the long inter-row bridges (arrows). Also the peripheral MTs appear partially disassembled (large arrowheads). R, microtubular rod; v, vane. Bar: 0.5 μm. Insert: Detail of $Ca^{2\oplus}$-treated microtubular rod revealing partial disintegration of bridging structures (arrows) and MTs (large arrowhead). Bar: 0.1 μm. 4b: Detail of microtubular rod after short saponin-lysis without subsequent extraction. Note integrity of the short (arrowheads) and long bridging structures (arrows). 4c: Detail of the untreated microtubular rod as a control. The comparison with the saponin-lysed rod (Fig. 4b) shows the structural integrity of the latter. Bars: 0.1 μm.

58 and/or saponin lysis respectively after PEM-extraction with or without $Ca^{2\oplus}$. Surprisingly, depending somewhat from the lysis conditions applied, not only the MTs of the anterior siphon part revealed now clear Glu-positivity, but the whole siphon complex stained brillantly (Fig. 3g). Apparently, the staining result shows a clear time dependency when using Brij 58/PEM-lysis and extraction conditions: Short time lysis without subsequent PEM-extraction yielded no staining at all (Fig. 3e), whereas a 5 min or even 10 min PEM-treatment enhanced the staining reaction considerably (Fig. 3f and 3 g). In contrast to the Brij 58-lysis, in saponin pretreated cells the siphon-MTs reacted always Glu-positive irrespective of the variations in PEM-extraction (Fig. 3i). Only in simultaneously saponin-permeabilized and fixed organisms, no Glu-staining was observed (Fig. 3h), whereas Tyr-stained controls (Fig. 3j) always yielded a positive reaction (see Table I).

These results clearly suggest that the MTs comprising the feeding organelle represent tubulin polymers containing almost equal amounts of the Glu- and Tyr-tubulin modification and that glutamylation and tyrosination of tubulin appeared early in the history of primitive eukaryotic cells. This situation is indeed somewhat aberrant, since most IIF-studies showed that MT-subpopulations exist which differ significantly in their Tyr/Glu-proportion (Geuens *et al.*, 1986; Greer and Rosenbaum, 1989). However, quantitative immun electron microscopy on the other hand has indeed shown that all so called Glu-MTs also contain to some extent Tyr-tubulin (and vice versa) and that the level of Glu- or Tyr-tubulin did not vary appreciably along individual MTs (Geuens *et al.*, 1986). In most cases, the sensitivity of the IIF-method is believed to be insufficient for the detection of somewhat differing amounts of the two tubulin modifications. In the case described, there is a rare example of an MT-organelle, sharing almost equal amounts of the two tubulin modifications.

Nevertheless, the question arises, why the siphon structure is usually not reactive with the Glu-antibody under conventional conditions. This phenomenon deserves special attention since in some cases described, an identic situation was evident. Certain cultured fibroblasts reveal no Glu-microtubules detectable by immunofluorescence, although they have been shown to exhibit stable MTs (Webster *et al.*, 1987) and it was also found that the marginal band of toad erythrocytes – an unusual stable array of MTs – contains no detectable Glu-tubulin at all (Gundersen and Bulinski, 1986). Similarly, Alfa and Hyams (1991) failed to detect any Glu-tubulin in *Schizosaccharomyces pombe.*

These results either suggest that the tyrosination/detyrosination cycle of α-tubulin may not extend to all cell types or perhaps, more likely, the epitopes of the Glu-modification may not be freely accessible in all cases since a distinct MAP-arrangement prevents the binding reaction. This could be in agreement with Sherwin *et al.* (1987), demonstrating in trypanosomes a correlation of crossbridge formation and the detyrosination state of their MTs.

Table I. Labelling results of *E. sulcatum* MT-structures with the antibody ID5 and YL1/2 after different lysis/extraction conditions. Abbreviations: Siphon, feeding apparatus; Pell. MTs, subpellicular microtubules; Flag, flagella; Nucl., nucleus. 0', Lysis without subsequent extraction; 5'/10', extraction-intervals: +Ca = PEM-buffer including calcium, -Ca = PEM-buffer without calcium.
Marking intensities: + = intensive; ± = slight; – = bare; 0 = no labelling. * = nuclear envelope.

		ID5				YL1/2			
Treatment		Siphon	Pell. MTs	Flag.	Nucl.	Siphon	Pell. MTs	Flag.	Nucl.
Simultaneously fixed and permeabilized cells	Triton X-100	0	+	+	+	+	±	+	–
	Saponin	0	+	+	+*	+	±	±	0
Brij 58-lysed cells (+Ca)	0'	0	+	+	+	+	–	–	–
	5'	±	0	0	+	+	0	0	–
	10'	+	0	0	+	+	0	0	–
Brij 58-lysed cells (-Ca)	10'	+	+	+	–				
Saponin-lysed cells (-Ca)	0'/5'/10'	+	+	+	±	+	±	±	0

In the electron microscope, the arrangement of crossbridging MAPs, although appearing somewhat deranged in Brij 58-treated *Entosiphon*, seems not so severly affected that a complete release of formerly masked Glu-epitopes must be a consequence. In the contrary, especially in the saponin-lysed *Entosiphon* cells, where Glu-positivity was achieved without extended extraction, the ultrastructure of the MAP-arrangement within the microtubular rods remains remarkably intact (compare Fig. 4b of a saponin-lysed cell with Fig. 4c of an untreated control), so that a presumed removal of crossbridging structures is obviously not the explanation for the successful reaction. Although, it is not completely to deny that there may be an influence on the accessibility of the Glu-epitopes, it appears more likely that the detergents used – especially saponin – produce conformational changes in the tubulin polymer itself.

In summary, the observations described, suggest that, when testing the occurrence of certain modified tubulins, one should be extremely careful in the case of a negative result, since such epitopes may be masked and not always be freely accessible for the antibody tested.

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