Cell Division of *Giardia intestinalis*: Flagellar Developmental Cycle Involves Transformation and Exchange of Flagella between Mastigonts of a Diplomonad Cell†

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*Giardia intestinalis* is a binucleated diplomonad possessing four pairs of flagella of distinct location and function. Its pathogenic potential depends on the integrity of a complex microtubular cytoskeleton that undergoes a profound but poorly understood reorganization during cell division. We examined the cell division of *G. intestinalis* with the aid of light and electron microscopy and immunofluorescence methods and present here new observations on the reorganization of the flagellar apparatus in the dividing *Giardia*. Our results demonstrated the presence of a flagellar maturation process during which the flagella migrate, assume different position, and transform to different flagellar types in progeny until their maturation is completed. For each newly assembled flagellum it takes three cell cycles to become mature. The mature flagellum of *Giardia* is the caudal one that possesses a privileged basal body at which the microtubules of the adhesive disk nucleate. In contrast to generally accepted assumption that each of the two diplomonad mastigonts develops separately, we found that they are developmentally linked, exchanging their cytoskeletal components at the early phase of mitosis. The presence of the flagellar maturation process in a metamonad protist *Giardia* suggests that the basal body or centriole maturation is a universal phenomenon that may represent one of the core processes in a eukaryotic cell.

The flagella of eukaryotic protists undergo a remarkable morphogenetic transformation called flagellar developmental cycle, whereby a flagellum passes through a maturation process before assuming its final position in the cell. In contrast to nucleus and some other organelles, which complete their development in a single cell cycle, it is now evident that in many groups of protists flagella require more than one cell cycle to mature (2, 26). In this respect, the behavior of flagellar structures in unicellular eukaryotes closely resembles that of centrioles in animal cells (3, 25).

On division of a flagellate, each daughter cell receives one half of parent flagella and/or basal bodies, while the other half arise de novo. This semiconservative distribution has been known for a long time (reviewed in reference 14). However, until a pioneering study of Melkonian et al. (24) it was not clear how unicellular protists maintain the structural and functional heterogeneity of their flagellar apparatus during division. The observations of Melkonian et al. (24) on the biflagellate heterokont green alga *Nephroselmis olivaceum* provided the first evidence that the heterogeneous flagellar apparatus is conserved in progeny through transformation of a flagellar type during cell division and that a newly formed flagellum requires more than one cell cycle to complete its development. The flagellar transformation has been later found in other groups of unicellular algae (reviewed in reference 2) and in representatives of other taxonomic groups of flagellated free-living protists (reviewed in reference 26). There are, however, several major groups of protozoan flagellates where mechanisms of development and maintenance of their heterogeneous flagellar systems are virtually unknown. Among these are Metamonada (12), a phylum of multiflagellated protozoa, including predominantly parasitic or symbiotic organisms such as trichomonads, diplomonads, and retortamonads. These organisms share in common primarily tetrakont arrangement of the flagellar apparatus with one recurrent and three anterior or anterolateral flagella, the number of which can be secondarily either reduced or increased (12).

The subject of the present study is the flagellar development of the parasitic diplomonad *Giardia intestinalis*, an intestinal pathogen of humans and some other mammals (for a review, see reference 23). In humans this parasite colonizes upper parts of the small intestine, where it reversibly adheres to the mucosa along the sides of villi, causing diarrhea that affects millions of people worldwide each year. The interphase cell of the *Giardia* trophozoite is binucleate with four pairs of basal bodies arranged in two clusters (tetrads). The left and the right tetrads are both situated in the midline between the anterior poles of the two nuclei side by side along the longitudinal cell axis (7), thus conforming to axial biradial symmetry typical for diplomonads (8). Each basal body subtends a flagellum; there are no barren or probasal bodies in *Giardia*. Due to specific arrangements of basal bodies and the presence of large intracytoplasmic portions of flagellar axonemes, all flagella are directed backward: two emerge ventrally, two emerge anterolaterally, two emerge posterolaterally, and two emerge caudally. Each tetrad of basal bodies together with pertinent flagella,
two microtubular roots, and some other fibrillar appendages form a structural unit called mastigont. The complexity of the *Giardia* cytoskeleton is further complicated with cytoskeletal components of the ventral adhesive disk (17), an essential organelle that mediates adhesion of *Giardia* to both the host mucosa and artificial substrates. The disk microtubules are modified homologues of the supranuclear fiber, one of the flagellar microtubular roots, characteristic for Diplomonadida (8, 9).

The replication of the *Giardia* trophozoites is far from being understood (1, 16). It is generally accepted that the flagellate multiplies by an asexual binary fission, in course of which the eight parent flagella are equally segregated between the two daughter cells, and four complementary flagella arise de novo in each. A unified view does not exist regarding the mitosis and behavior of the two mastigonts during division (13, 18, 23, 33). Also, the assembly of daughter ventral disks is poorly understood (reviewed in reference 16), and contradictory interpretations of the plane of *Giardia* cytokinesis have been repeatedly published (4, 18, 19, 21, 32, 33, 36).

There are at least two major obstacles limiting studies on *Giardia* division: (i) the complexity of the microtubular cytoskeleton (16, 23) that makes dividing structures incomprehensible and (i) the small number of dividing cells in cultures (1 to 2% in the logarithmic phase of growth) (21, 33) and a lack of efficient synchronization procedures (1). We have partially overcome these difficulties by developing a technique that increases the number of mitotic cells in cultured *Giardia* populations and by the use of monoclonal antibodies, allowing discrimination of parent and de novo-formed microtubules. Here we present new data on the cell division of *Giardia* focused on the reorganization of microtubular structures during the division, and we describe for the first time the flagellar maturation process in this tetrakont octoflagellated diplomonad. Our results are based on conventional and confocal microscopy, selective immunofluorescence, and scanning and transmission electron microscopy.

**MATERIALS AND METHODS**

**Culture conditions.** *Giardia intestinalis*, strain Portland (ATCC 3088), an original human isolate provided by E. A. Meyer (Oregon Health Science University, Portland), was used. Axenic cultures were routinely maintained in a modified TYI-S-33 medium supplemented with bovine bile (22).

For immunofluorescence and electron microscopy experiments, a cultured population was enriched with mitotic cells (Fig. 1) according to a protocol based on the specific effect of albendazole on *Giardia* cells. Briefly, mid-log-phase trophozoites forming nearly a confluent monolayer were exposed to 100 ng of albendazole (Sigma)/ml in fresh TYI-S-33 medium for 7 h. The culture medium plus free-swimming albendazole-affected cells were then quickly discarded, and the adherent nonaffected cells were immediately overlaid with the fresh medium plus free-swimming albendazole-affected cells were then quickly discarded, and the adherent nonaffected cells were immediately overlaid with the fresh medium without drug. For immunofluorescence and scanning electron microscopy, the cultures were then chilled on ice-water bath for 5 min to detach the cells. The suspension was immediately injected into a 3-mm-deep perfusion anaerobic chamber (Sigma), followed by incubation at 37°C for 10 min. During this period the cells attached and entered mitosis. For transmission electron microscopy, the cells were fixed while attached to a slide. Giemsa-stained cells were viewed from their dorsal sides to show the typical orientation (arrows) of the median body (MB). Bar, 10 μm.

**Monoclonal antibodies.** The monoclonal antibody 6-11B-1 (immunoglobulin G [IgG]) against acetylated amino-terminal peptides of α-tubulin (Sigma) was used as a marker of parent flagellar kinetosomes/axonemes and the median body (11). These antibodies were selected on the basis of preliminary experiments with a panel of 23 monoclonal antibodies against different epitopes on N and C termini of α- and β-tubulins and against known posttranslational modifications of tubulin. Fluorescein isothiocyanate- and Texas Red-conjugated anti-mouse IgG antibodies were purchased from Sigma and Vector, respectively.

**Immunofluorescence.** For immunofluorescence, a method described previously was used (20). The cells attached to a glass coverslip forming a bottom of the anaerobic chamber were fixed with ice-cold methanol (at −20°C for 5 min), permeabilized with ice-cold acetone (at −20°C for next 5 min), and air dried. The following steps were performed at ambient temperature. After rehydration (10 min) with phosphate-buffered saline (PBS; pH 7.4), the cells were blocked with 3% (wt/vol) bovine serum albumin in PBS for the next 30 min, followed by a 1-h incubation with monoclonal antibody diluted 1:100 (6-11B-1) or 1:1,000 (AXO 49) with 2% (wt/vol) bovine serum albumin in PBS. After a wash in PBS (three times, 5 min each time), the cells were incubated with diluted fluorescein isothiocyanate- and Texas Red-conjugated anti-mouse IgG antibodies purchased from Sigma and Vector, respectively.

**Transmission electron microscopy.** The suspended cells were fixed with solution containing 2.5% glutaraldehyde (Polysciences) and 5 mM CaCl₂ in 0.1 M cacodylate buffer (pH 7.2) for 2 h at room temperature, washed three times with PBS, and postfixed with 1% OsO₄, 0.8% potassium ferricyanide–5 mM CaCl₂ in 0.1 M cacodylate buffer. After a wash with an excess volume of PBS, the fixed cells were dehydrated in acetone and embedded in Epon (Poly/Bed 812; Polysciences). The ultrathin sections were stained with lead citrate and uranyl acetate and examined with a JEOL 1010 electron microscope.

**Scanning electron microscopy.** For scanning electron microscopy, the cells attached to glass coverslips were fixed with 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) for 30 min at 4°C, washed three times with the same buffer.
containing 5% sucrose, postfixed with 1% OsO4 in 0.1 M cacodylate buffer for 30 min at 4°C, washed several times with PBS, dehydrated in graded series of ethanol, critical point dried, coated with gold, and observed with a JEOL 6300 scanning electron microscope.

RESULTS

Introductory remarks. (i) Flagellar pairs and basal body pairs. A prerequisite to comprehension of basal bodies and flagella rearrangements in a dividing Giardia trophozoite is the understanding of their basic arrangements in the interphase cell (Fig. 2). It is important to note that the flagellar pairs do not conform to the basal body pairs. The flagellar pairs are formed by flagella of the same type, i.e., of the same function and location in each diplomonad mastigont (Fig. 2a). Basal bodies of all flagellar axonemes are clustered in the midline between nuclei in two sets of four (tetrads) (Fig. 2, insets), each set comprising one basal body of each flagellar pair. Basal body pairs result from pairing within each tetrad. Thus, each basal body pair is formed by basal bodies of different flagellar types. Moreover, due to incomplete axial symmetry of Giardia cell (7), the left and right tetrads differ in the composition of their basal body pairs (Fig. 2b).

(ii) Plane of observation. For understanding the sequence of morphological changes occurring during the Giardia division, standard orientation of observed cells is essential. Since Giardia trophozoites start to divide while attached to a substratum by ventral disk, we describe all events of the process as seen in cells viewed from their dorsal side. Also, all figures in the present study are oriented in the same way. After immunofluorescence staining of interphase and early mitotic cells, the dorsal view is readily discernible by the topology of the median body that is located slightly oblique to the longitudinal body axis. As seen from above, the right end of this structure is in a more anterior position than the left one (Fig. 1, 3, 5a, and 8a). In the late mitosis, the dorsal view is discernible by the parent disk cytoskeleton overlapping on the right anterior side (Fig. 8b, arrowhead) and by typical arrangement of the left posterolateral flagellar axoneme (Fig. 3 and 5d to g).

Flagella. Eight flagella of the parent cell persisted during the cell division. They were transformed and distributed between...
two daughter cells in a semiconservative manner so that each progeny received a different set of four parent flagella. In each daughter cell, the flagella of both posterolateral and ventral pairs arose during mitosis de novo. The progress of the parent daughter cell, the flagella of both posterolateral and ventral progeny received a different set of four parent flagella. In each two daughter cells in a semiconservative manner so that each

To visualize the behavior of basal bodies and intracytoplasmic portions of axonemes of the anterolateral flagella that enabled the migration described above, the monoclonal antibody AXO 49 to polyglycylated carboxy-terminal peptides of α- and β-tubulin was used. In immunofluorescence experiments, this antibody specifically labeled basal bodies and axonemes of the parent cell flagella but not those of newly assembled ones.

Antibody staining revealed the reorientation of the anterolateral basal bodies and the striking migration of the basal body/axonemes inside the cell (Fig. 5b to h). A subtle curving of the bases of the anteriorly directed axonemes of the anterolateral flagella toward the midline (Fig. 5b) marked the initiation of the division. The basal body located on the right side of the interphase basal body complex then turned dorsally and, curving to the left, moved toward the left side of the cell (Fig. 3[AFL] and 5c and d). The axoneme was thus pulled deeper inside the cell, as confirmed by transmission electron microscopy (see Fig. 7b). The left basal body or axoneme underwent a similar, but opposite migration moving toward the right side of the cell (Fig. 3[AFL] and 5c and d). The lateral migration of their intracytoplasmic portions toward opposite sides led to a disjunction of anterior axonemal crossing and to the exchange of positions of both their basal bodies and their exits, as seen by scanning electron microscopy (see above).

The antibody staining also revealed intracellular reorientation and migration of basal bodies and proximal parts of axonemes of other parent flagella that was not apparent by scanning electron microscopy. Those of the right posterolateral and the left ventral flagella migrated to opposite body halves with respect to their interphase position (Fig. 5Ad and e and 6). Those of the left posterolateral and the right ventral flagella, as well as those of the left and right caudal ones, remained in their interphase body half, curving laterally (Fig. 5Ac to e and 6b to d, arrows). The interphase basal body pairing (left anterolateral-left ventral, left caudal-left posterolateral, right ventral-right caudal, right anterolateral-right posterolateral; Fig. 2) was maintained during these movements (Fig. 5Ad and e and 7c). Owing to migration, the parent basal bodies were partitioned so that the proximal parts of the right anterolateral, the right and the left posterolateral, and the left caudal parent flagella were located in the left anterior half of the parent cell. Those of the left anterolateral, the left and the right ventral, and the right caudal flagella were in the right anterior half (Fig. 5Ad and e; see videos S1 and S2 in the supplemental material). Intracytoplasmic migration of the posterolateral, ventral, and caudal basal bodies/axonemes did not affect the positions of exits of their respective flagella (Fig. 5Ab to e). Transmission electron microscopy confirmed the intracytoplasmic reorienta-
FIG. 5. Reorganization and transformation of the parent flagella during the cell division of *Giardia*, as shown by immunofluorescence. The selection of representative micrographs is based on a comparative study of more than 500 documented cells. Cells were labeled with the antibody AXO 49 selectively staining the parent but not the newly arising flagella. (A) Temporal sequence of flagellar relocation in dividing *Giardia*. Nuclei briefly counterstained with propidium iodide were pseudocolored in red. (B) Same sequence of micrographs as in panel A with color-coded flagellar axonemes: anterolateral (red), posterolateral (green), ventral (blue), and caudal (white). The sequence starts with an interphase cell (a) and follows gradual migration and transformation of parent flagella (b to h) up to their final distribution between the two daughter cells (i). In each progeny, the transformed parent flagella form the anterolateral and caudal flagellar pairs. For a detailed explanation, see the text. Arrowheads indicate the left posterolateral flagellum, which serves as a marker for the correct orientation of a dividing cell. Focal thinning of the anterolateral axonemes (white arrows) indicates the site of flagellum exit. The plane of *Giardia* cytokinesis is indicated by a red arrow (g). Bar, 10 µm.
tion of the parent flagellar structures (Fig. 7b and c). As the division proceeded further, reorganization of the parent flagella became more pronounced (Fig. 5Af to h; see video S3 in the supplemental material). The basal bodies forming the interphase pairs separated from each other. This enabled the most surprising changes at this phase, that is, the transformation of the parent posterolateral and ventral flagella to daughter anterolateral flagellar pairs. In the left body half, the bases of the two parent posterolateral flagella crossed each other to form an origin of anterior crossing of the daughter anterolateral axonemes through further reorirentation of their basal bodies (Fig. 5Af to h). In the right body half, a slight crossing of the bases of the parent ventral flagellar axonemes also resulted from advancing reorientation of the basal bodies (Fig. 5Af to h), during which the proximal part of the right ventral axoneme flips (Fig. 5Af and g).

(ii) Functional transformation of parent flagella during cell division. Consequent to flagellar reorganization during divi-
sion, each of the two daughter cells acquired a heterogeneous set of the parent flagella (compare the color-coded axonemes in Fig. 5Ba and i). As seen from the dorsal side, the left offspring received one anterolateral, one caudal, and two posterolateral parent flagella, whereas the right daughter cell received one anterolateral, one caudal, and two ventral parent flagella (Fig. 5Bh and i). The pairs of the parent posterolateral and ventral flagella transformed through reorientation to pairs of anterolateral flagella in the left and right daughter cells, respectively (Fig. 5Bi to i). In each daughter cell, the parent anterolateral flagellum developed through reorientation during cell division into a caudal flagellum and joined the parent caudal flagellum, thus completing the daughter caudal flagellar pair (Fig. 5Bf to i).

(iii) Newly assembled daughter flagella. The pairs of ventral and posterolateral flagella were assembled de novo in each daughter progeny. Thus, each basal body pair in the progeny and consequently, in the interphase cell, consisted of an old parent basal body and a new one. Because the monoclonal antibody AXO 49 did not recognize newly arising flagella (Fig. 5Ai), we used the monoclonal antibody 6-11B-1 against acetylated N terminus of α-tubulin. In Giardia, this antibody labeled both parent and newly assembled flagella, along with parent and daughter ventral disks, and the median body (Fig. 8). New flagella were detected shortly after the initial reorientation and the lateral partitioning of the parent flagellar structures (Fig. 8b and c). In each cell half, four flagellar axonemes arose close to the four parent basal bodies from newly assembled ones. The intracytoplasmic axonemes from both cell sides were directed toward the midline of the dividing cell. Elongation of the intracytoplasmic axonemes continued throughout division so that, before detachment of the dividing cell (data not shown), they reached the cell membrane but did not emerge as free flagella (Fig. 8f).

Flagellar maturation. As a consequence of flagellar transformation during Giardia division, the resulting interphase cell possessed flagella of different ages and functions. The flagella of ventral and posterolateral pairs represented the youngest flagella being assembled de novo during every division. The flagella of anterolateral pair were a generation older: in every division, they were formed by transformation of either the ventral or the posterolateral flagellar pairs. The caudal pair was composed of the oldest flagella. However, the caudal pair consisted of flagella of unequal age in contrast to the other pairs within the cell. The flagellum coming from transformation of the parent anterolateral flagellum was two generations...
old, whereas the other one was at least one generation older. Only this flagellum represented a true mature flagellum (flagellum 1 according to the nomenclature of Beech et al. [2]). These results show that in *Giardia* the basal body/flagellar maturation is spread over three successive cell cycles. During this process each newly assembled flagellum undergoes three transformation events to become the mature caudal flagellum (Fig. 9).

**DISCUSSION**

Observations on the cell division of a unicellular parasitic eukaryote *Giardia intestinalis* presented in the present study revealed that the two mastigonts of this diplomonad do not reconstitute separately during the division but act as a single unit exchanging their cytoskeletal components. Our findings showed reorientation, migration, and functional transformation of the parent basal bodies and flagella, thus demonstrating the presence of a flagellar developmental cycle as described by Melkonian and coworkers (20). As is apparent from these results, each newly assembled flagellum of *Giardia* requires three cell cycles to mature. To our knowledge, this is the first direct evidence on the flagellar maturation process among metamonads.

**Structural recombination between the diplomonad mastigonts of *Giardia*.** As observed earlier (33), a division of *Giardia* is in accordance to the semiconservative principle of partitioning parent flagella. This is apparently a universal phenomenon among flagellated eukaryotes dividing in vegetative stage (14). During the division each daughter *Giardia* receives four flagella from the parent cell that are supplemented to the full eight-flagellate set by flagella arisen de novo. Due to a plane of *Giardia* cytokinesis that cleaves the dividing cell between segregated mastigonts, it has been incorrectly assumed that each of the daughter cell inherits one of the two parent mastigonts, i.e., the two basal body pairs with pertinent appendages of the same mastigont (18, 23, 33). By using flagellum-specific immunostaining we found, however, that before mastigont separation a half of each flagellum components migrates to opposite cell side, whereas the other half retains its interphase position. Consequently, the parent set inherited by progeny is composed of a half of each parent mastigont. Two diplomonad flagellar apparatuses thus undergo in common a single maturation program and can be characterized as a developmentally linked double tetrakont. These findings are in contrast to current understanding of the cell organization of diplomonads, which are interpreted as double cells equipped with a duplicated set of the same unit of basal bodies bearing flagella (26, 31). The two mastigonts of diplomonads are considered developmentally separated, and the evolutionary origin of diplomonads from mononuclear tetrakont ancestors by heterochrony of cell division has been proposed (30). It remains to be investigated whether the exchange between mastigonts also occurs in other diplomonad genera such as *Hexamita*, *Spironucleus*, and *Octomitus*, which do not show any left-right asymmetry (8), or whether it has evolved specifically in *Giardia* as a consequence of regulating the organization of the ventral disk cytoskeleton.

**Flagellar transformation.** We further demonstrated that during the exchange between mastigonts, all but two parent flagella transform to another flagellar types. This transformation is mediated by reorientation and migration of the pairs of basal bodies through which the respective flagella change their position and function. A similar mode of flagellar transformation has been reported in octoflagellated (27) and biflagellated (20) unicellular algae, as well as in biflagellated bodonids (5), or in the process of probasal body maturation in the trypomastigotes of unflagellated fish trypanosomes (35). During the transformation process in *Giardia*, the parent anterolateral flagella become caudal, whereas both parent posterolateral and ventral flagella become anterolateral in daughter cells. The two parent caudal flagella do not transform to a distinct type but differ in age. The right flagellum is younger, becoming the left one in a daughter cell of the next generation, while the left caudal flagellum represents the oldest, mature flagellum that does not transform further. These observations are consistent with findings obtained with other flagellated protists investigated so far, thus confirming a general rule that a mature basal body and/or flagellum that has attained a genetically predetermined final stage of flagellar development does not transform in successive generations (2, 10). Also, partitioning of the two oldest flagella in *Giardia* conforms to a universal scheme of generational asymmetry as determined for partitioning of a flagellar pair in biflagellates (2, 10), as well as for a centriolar pair in vertebrate cells (3, 25). In all of these cells, one daughter progeny inherits the mature basal body or centriole, which is at least one cell cycle older than that inherited by the second progeny. Interestingly, the perikinetosomal area of the left caudal flagellum of *Giardia* has an important morphogenetic function, serving as the organizing center for the ventral adhesive disk microtubules (17). Thus, the competence to nucleate the disk microtubules can serve as a marker that defines the mature basal body in *Giardia*.

**Developmental asymmetry of the microtubular roots of caudal flagella.** Generational asymmetry of the caudal flagella may be essential for maintaining developmental left-right asymmetry of the interphase *Giardia* cell, manifested phenotypically in ultrastructural organization of the ventral adhesive disk. The basal layer of the disk skeleton is composed of microtubules derived from a basal body root that is asymmetrically developed (7, 8, 15). Of the eight basal bodies present in interphase *Giardia*, only the basal bodies of caudal flagella carry microtubular roots. There are two roots adjacent to each of the two basal bodies: one directed backward (funis) and the other directed anteriorly. Of these, only the anterior roots are developed asymmetrically. The left anterior root, belonging to the mature basal body, is extensively developed to form the ventral disk microtubules, whereas the right one is rudimentary (7, 15), representing a precursor that will develop into the disk armature in the next generation when the basal body becomes mature. The two basal bodies and/or their perikinetosomal matrix apparently differ in their functional competence. The microtubules of the adhesive disk always nucleate at the left caudal basal body and always wound in the same clockwise direction, thus determining the left-right asymmetry of the cell. The importance of maturation for a functional competence has been documented in centrioles. As demonstrated in animal cells, the mature and the immature centriole of a centriolar pair differ in their respective contributions to form interphase microtubules (29). Similarly, in biflagellates the mature basal body usually carries the “mature” microtubular root, which is
often associated with specific cell functions such as cytostome enforcement in bodonids (26). The available data suggest that the basal body and/or centriole maturation is a universal, evolutionary conserved phenomenon representing one of the core attributes of a eukaryotic cell.

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