Naegleria gruberi De Novo Basal Body Assembly Occurs via Stepwise Incorporation of Conserved Proteins

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Centrioles and basal bodies are discrete structures composed of a cylinder of nine microtubule triplets and associated proteins. Metazoan centrioles can be found at mitotic spindle poles and are called basal bodies when used to organize microtubules to form the core structure of flagella. Naegleria gruberi, a unicellular eukaryote, grows as an amoeba that lacks a cytoplasmic microtubule cytoskeleton. When stressed, Naegleria rapidly (and synchronously) differentiates into a flagellate, forming a complete cytoplasmic cytoskeleton de novo, including two basal bodies and flagella. Here, we show that Naegleria has genes encoding conserved centriole proteins. Using novel antibodies, we describe the localization of three centrosomal protein homologs (SAS-6, γ-tubulin, and centrin-1) during the assembly of the flagellate microtubule cytoskeleton. We also used these antibodies to show that Naegleria expresses the proteins in the same order as their incorporation into basal bodies, with SAS-6 localizing first, followed by centrin and finally γ-tubulin. The similarities between basal body assembly in Naegleria and centriole assembly in animals indicate that mechanisms of assembly, as well as structure, have been conserved throughout eukaryotic evolution.

The beautiful and enigmatic pinwheel structures of centrioles and basal bodies have captured the imaginations of cell biologists for over a century. These small (~1-μm) organelles are composed largely of a cylinder of nine microtubule triplets (11). The surrounding amorphous material harbors the microtubule-organizing activities of the centrosome, placing centrioles at the hub of the microtubule cytoskeleton. Metazoan centrosomes define mitotic spindle poles, and their centrioles are called basal bodies when used to form cilia (29). Moreover, in 1900 Meeves showed in a series of classical experiments that centrosomes define mitotic spindle poles, and their centrioles are interconvertible structures (34). Centrioles must replicate exactly once per cell cycle, as duplication occurs synchronously, with approximately 90% of cells growing synchronously, with approximately 90% of cells growing visible flagella in a 15-min window (\(T_{50} = 65\) min after initiation of differentiation). As part of this differentiation, Naegleria has been shown to assemble the pinwheel structure of the basal bodies de novo, about 10 min before flagella are seen (11).

Two centrosomal proteins that have been studied during Naegleria differentiation are centrin and γ-tubulin. Centrin is a calcium-binding phosphoprotein that is an integral component of the wall and lumen of basal bodies and of the pericentriolar lattice in many organisms (4, 19). During differentiation, Naegleria induces synthesis of centrin protein, which then localizes specifically to basal body structures throughout differentiation (18). γ-Tubulin is a general microtubule nucleation factor that localizes to microtubule-organizing centers (MTOCs) of many types. Surprisingly, Naegleria’s γ-tubulin homolog has been reported to localize to basal body precursor complexes and then move to the other end of the cell before disappearing completely (32).

A third protein that has come under recent scrutiny for its role in centriole duplication is SAS-6, a functionally conserved coiled-coil protein required for the formation of diverse basal body precursor structures (7, 21–23, 31). In Caenorhabditis elegans and Drosophila melanogaster, SAS-6 is recruited to S phase to form the “central tube,” a cylindrical basal body precursor that lacks microtubules (22, 23). SAS-6 is also required for the formation of the flat ring or cartwheel with nine radiating spokes, which is the first structure to be formed in the Chlamydomonas basal body (21).

To determine if Naegleria is likely to have typical basal body components, we identified conserved basal body genes in the Naegleria genome. We also made antibodies to and localized Naegleria’s homologs of SAS-6 and γ-tubulin. Finally, we have determined the order of expression and incorporation of these proteins, as well as centrin, during Naegleria de novo basal body assembly.

MATERIALS AND METHODS

Identification of Naegleria basal body genes. To determine whether Naegleria’s basal bodies are formed from canonical proteins, known components were iden-
Naegleria (strain NEG) amoebae were grown on solid medium in association with Klebsiella pneumoniae and differentiated as described previously (9). Differentiation and visualization of the cytoskeleton. Naegleria gruberi strain NEG amoebae were grown on solid medium in association with K. pneumoniae and differentiated as described previously (9). Differentiation was tracked via the appearance of flagella stained with LUGO's iodine (10) and visualized using an α-tubulin phase objective.

Cells taken at each time point were added to an equal volume of fixation buffer (125 mM sucrose, 50 mM sodium phosphate [pH 7.2] with 2% paraformaldehyde) and fixed in suspension for 5 min. Cells were then smeared onto polyethyleneimine-coated coverslips and allowed to continue fixing for 15 min. The slides were washed three times in Detector Block and three times with PEM supplemented with 0.01% Triton X-100 and mounted with ProLong Gold antifade reagent with DAPI (4′,6-diamidino-2-phenylindole) (Invitrogen). Centrin-1, γ-tubulin, and SAS-6 foci were counted from at least 100 cells at each time point using an Olympus BX51 fluorescence microscope with an Olympus PlanApo 100 (numerical aperture [NA], 1.35) oil immersion objective.

Fluorescence deconvolution microscopy. Images were collected with SoftWorx image acquisition software (Applied Precision, Issaquah, WA) on an Olympus IX70 wide-field inverted fluorescence microscope with an Olympus PlanApo 100 (NA, 1.35) oil immersion objective and Photometrics charge-coupled device (CCD) CH350 camera (Roper Scientific, Tucson, AZ). Image stacks were deconvolved with the SoftWorx deconvolution software and flattened as maximum projections (Applied Precision, Issaquah, WA).

**RESULTS**

**Naegleria has canonical basal body genes.** Using the Naegleria genome sequence (http://genome.jgi-psf.org/Naegl1/Naegl1.home.html), we identified many conserved basal body genes (Table 1). These proteins include cartwheel components (POC1, SAS-6, and BLD10) and blade components (δ-tubulin and RIB43), as well as proteins previously localized to the centriole/basal body lumen (SAS-6 and γ-tubulin) and proteins that make up centriole appendages in other systems (SF-assemblin and centrins) (localizations are reviewed in references 16 and 20). Naegleria’s genome encodes two centrins: the previously characterized centrin (which we followed in this study) (19) that is a homolog of human centrin-1, and a second centrin that represents a homolog of human centrin-3 and yeast Cdc31p. Surprisingly, we did not find evidence of a homolog of pericentrin, despite a report of localization of this protein in **Naegleria** using heterologous antibodies (32). The presence of many known basal body genes indicates that although **Naegleria**’s basal bodies are transient structures, they are likely canonical in composition as well as structure.

**Order of centrosomal protein induction.** As we were unable to reliably detect Naegleria γ-tubulin using heterologous antibodies for immunofluorescence, we raised and affinity purified a polyclonal antibody to a 97-amino-acid segment of the **Naegleria** γ-tubulin gene. To investigate early basal body as-
SAS-6, centrin, and γ-Tubulin were collected at 14 different time points between 0 and 90 min and used for Western blotting along with actin. Actin protein levels were used as a loading control. These blots indicate the following order of protein induction to differentiate: SAS-6, centrin-1, and finally γ-Tubulin. For panels A to C, lanes labeled “A” and “F” contain Naegleria flagellate extract immunoblots. (B) The previously described centrin-1 antibody recognizes a band of the correct size (20 kDa [19]) on Naegleria flagellate extract immunoblots. (C) The γ-Tubulin antibody produced for this study recognizes a band of the correct size (55 kDa) on Naegleria flagellate extract immunoblots. (D) Cell extracts were collected at 14 different time points between 0 and 90 min. Western blots were probed using antibodies against SAS-6, centrin, and γ-Tubulin. Actin was used as a loading control. These blots indicate the following order of protein induction to detectable levels: SAS-6, centrin, and finally γ-Tubulin.

FIG. 1. Induction of centrosome proteins during differentiation. For panels A to C, lanes labeled “A” and “F” contain Naegleria protein extracts from amoebae and flagellates, respectively. (A) The SAS-6 antibody produced for this study recognizes a band of the correct size (74 kDa) on Naegleria flagellate extract immunoblots. (B) The previously described centrin-1 antibody recognizes a band of the correct size (20 kDa) on Naegleria flagellate extract immunoblots. (C) The γ-Tubulin antibody produced for this study recognizes a band of the correct size (55 kDa) on Naegleria flagellate extract immunoblots. (D) Cell extracts were collected at 14 different time points between 0 and 90 min. Western blots were probed using antibodies against SAS-6, centrin, and γ-Tubulin. Actin was used as a loading control. These blots indicate the following order of protein induction to detectable levels: SAS-6, centrin, and finally γ-Tubulin.

Assembly events, we also raised a polyclonal antibody against the Naegleria SAS-6 homolog. The resulting SAS-6 (Fig. 1A) and γ-Tubulin (Fig. 1C) antibodies each recognize a single band of the appropriate size (74 and 55 kDa, respectively) on immunoblots of Naegleria flagellate extracts. These antibodies, as well as a published antibody that recognizes Naegleria centrin-1 (Fig. 1B) [19], were used to follow protein levels during differentiation (Fig. 1D). Cell extracts were collected at 14 different time points between 0 and 90 min and used for Western blotting along with actin. Actin protein levels were used as a loading control, as they remain constant throughout differentiation (33).

The first protein to reach detectable levels after induction of differentiation was SAS-6, with detectable protein by 5 min and maximum levels by 25 min. This is well before basal bodies are known to assemble (visible flagella appear by 65 min, and basal bodies form approximately 10 min prior [11]). Centrin-1 was the next protein to appear, being detectable by 20 min and reaching the maximum at 60 min (18). γ-Tubulin was not detectable until 60 min, when it appeared near maximum levels. All proteins remained at peak levels through the rest of the 90-min time period. In summary, basal body proteins are induced in the following order: SAS-6, centrin-1, and finally γ-Tubulin.

**Naegleria SAS-6 and γ-Tubulin localize to basal bodies throughout differentiation.** We also used our γ-Tubulin and SAS-6 antibodies to localize their target proteins during differentiation. As has been previously described, no microtubule- or centrin-1-containing structures were detected in interphase amoebae (19). Likewise, we found no γ-Tubulin-containing structures in interphase amoebae or cells early in differentiation. The spindles of mitotic amoebae were detectable with antitubulin antibodies, but mitotic amoebae did not contain any detectable centrin-1- or γ-Tubulin-containing structures (data not shown). γ-Tubulin foci appear before flagella assembly (by 55 min) and remain at the base of the flagella throughout differentiation (Fig. 2).

Like γ-Tubulin, SAS-6 is absent in Naegleria amoebae and early-stage flagellates (Fig. 3). However, staining with the anti-SAS-6 antibody revealed a single round focus that appeared by 40 min. SAS-6 was consistently located at the proximal ends (relative to centrin-1) of both early basal body structures and mature basal bodies, with flagella emerging from the centrin-1-positive distal ends in fully formed flagellates (Fig. 3). This localization pattern is similar to that seen in other systems, particularly the localization of SA6-6 to the cartwheel of Chlamydomonas (21) and to the proximal ends of both Tetrahymena basal bodies and animal centrioles (7, 31).

**Order of incorporation of SAS-6, centrin-1, and γ-Tubulin during assembly.** To determine the order of centrin-1, SAS-6, and γ-Tubulin incorporation into basal bodies, we performed immunofluorescence with each antibody at 5-min intervals during differentiation (Fig. 4). At least 100 cells per sample were scored for localization, and the times were normalized to the point at which 50% of cells have visible flagella (65 min). From these data, it is clear that SAS-6, centrin-1, and γ-Tubulin localize to basal bodies in the same order as protein induction; at $t = 30$ min, a wave of SAS-6 foci begins, followed by centrin-1 foci, which begin at $t = 35$ min, and finally γ-Tubulin foci at $t = 40$ min. Each of these proteins localizes well before centrioles form (approximately 10 min before visible flagella [11], or around 55 min).

It should be noted that although protein localization follows the same order as induction, γ-Tubulin is detectable via immunofluorescence at earlier time points than on immunoblots. This may be because the γ-Tubulin in ~20% of cells at 45 min is concentrated enough to be detectable by immunofluorescence, but when this γ-Tubulin is diluted with proteins from the remaining ~80% of cells (which do not have visible γ-Tubulin localization), the total γ-Tubulin protein concentration is not great enough to detect by Western blot.

**DISCUSSION**

Although centrioles and basal bodies have been studied for well over a hundred years, their components, their order of assembly, and the regulation of their number are still largely mysterious. For example, proteomic analysis of *Chlamydomonas and Tetrahymena* basal bodies (14, 16) and human centrosomes (2) suggests that these structures potentially contain hundreds of different proteins. However, we only know when a few of these proteins are incorporated into the structure (30). This is in part due to two obstacles. First, centriole replication in other eukaryotes occurs within an existing microtubule cytoskeleton (and frequently attached to a preexisting centriole), often obscuring which proteins are being used specifically for new centriole assembly. Second, de novo assembly in other systems can be assayed only in single cells or embryos, making gathering enough material for bulk analysis to determine order of incorporation difficult. Both of these obstacles are overcome...
by using *Naegleria* to analyze *de novo* basal body assembly. In this study, we have shown that by using antibodies to make a fine time course of protein localization, we can study the order of incorporation of any protein into *Naegleria* basal bodies with relative ease.

The antibodies that we have raised to the *Naegleria* SAS-6 and γ-tubulin homologs in this study have revealed conserved localizations for these key centriolar proteins. In particular, SAS-6 localizes to the proximal ends of basal bodies (relative to centrin-1), as seen in other systems (21, 31). Additionally, we observed that γ-tubulin is consistently localized to the basal bodies throughout differentiation. This result is in direct contrast to what has been previously described (32). We propose that the previous reports of *Naegleria* γ-tubulin foci moving to the opposite end of the cell from the MTOC after basal body assembly were likely an artifact of using heterologous antibodies. This is a particular problem for *Naegleria*, as we have observed several nonspecific antibodies that seem to localize to a round structure toward the posterior end of the cell (data not shown).

We have outlined a basal body assembly pathway for *Naegleria* by following the timing of localization of centriolar proteins. These results indicate that SAS-6 localizes first, then centrin-1, and finally γ-tubulin. The localization of SAS-6 before centrin-1 suggests that the *de novo* basal body assembly in *Naegleria* is similar to centriole assembly during templated duplication. For example, SAS-6 foci form early in S phase of human cells, before the localization of centrin-1 (31). SAS-6 is also required for proper assembly of the earliest basal body precursor structure in *Chlamydomonas*, the cartwheel (21). Given the observation that both humans and *Naegleria* incorporate SAS-6 before centrin-1 into centriolar structures and the evolutionary distance between these organisms (3), this stepwise mechanism is likely ancestral to all eukaryotes.

In contrast, it is difficult to compare the timing of γ-tubulin localization during templated centriole or basal body formation in other organisms to *de novo* basal body assembly in *Naegleria*, as the former occurs next to preexisting centrioles or basal bodies, environments already enriched in γ-tubulin, and the latter occurs during the initial stages of MTOC assembly. However, previous studies have shown that γ-tubulin is necessary for centriole assembly in a variety of organisms, including

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**FIG. 2.** *Naegleria* γ-tubulin localization during differentiation. *Naegleria* cells were fixed at 30, 45, 60, and 90 min during differentiation and stained with antibodies to γ-tubulin (green) and α-tubulin plus β-tubulin (red), with DNA shown in blue. No appreciable localization of γ-tubulin appears by 30 min. However, by 60 min two clear foci are present, which are localized to the base of the growing axonemes (highlighted by tubulin staining). Basal body structures are indicated by an arrow and are enlarged in the insets. The scale bar represents 10 μm.
animals (4) and ciliates (i.e., Paramecium [24] and Tetrahymena [26]). Here, we show that γ-tubulin localizes after incorporation of SAS-6 and centrin-1 but before the formation of recognizable basal bodies during Naegleria differentiation. Together, these data suggest that the initial stages of centriole assembly (the incorporation of centrin-1 and SAS-6) do not require detectable amounts of γ-tubulin. Rather, γ-tubulin is likely used during the subsequent formation of centriolar microtubules and later for the cortical microtubules that emanate from the basal body-containing MTOC.

We were surprised not to detect γ-tubulin in mitotic amoe-bae. Very little is known about Naegleria’s mitotic spindle, which is contained within a nuclear envelope (25) and represents the only microtubule structure in Naegleria amoebea. The Naegleria spindle uses an extremely divergent α-tubulin (6), which supports the hypothesis that Naegleria has two independent microtubule cytoskeletons (a divergent one used for the mitotic spindle, and a more canonical one is used in flagellates) (13). If this is true, the microtubule nucleation factors (including γ-tubulin) used in the Naegleria spindle may have correspondingly diverged and are no longer recognized by sequence (we can identify only one γ-tubulin gene in the genome) or by our antibody.

Naegleria has long been recognized for its remarkable ability to form an entire cytoplasmic microtubule cytoskeleton from scratch. Although Naegleria makes its basal bodies de novo, they are canonical in structure (4, 11). The presence of many known basal body genes argues that they are canonical in composition as well. This is in contrast to certain well-established model organisms that possess centrioles with modified ultrastructures or are missing otherwise well-conserved genes. For example, C. elegans centrioles have singlet microtubule blades (4) and lack centrin (5).

Naegleria’s synchronous de novo basal body assembly makes it a good system with which to study centriole assembly, particularly as it expresses proteins in the same order as it incor-

FIG. 3. Naegleria SAS-6 and centrin-1 localization during differentiation. Naegleria cells were fixed at 30, 45, 60, and 90 min during differentiation and stained with antibodies to SAS-6 (green), centrin (blue), and α-tubulin and β-tubulin (red), with DNA shown in gray. No appreciable localization of any of the proteins appears by 30 min. By 45 min, all bar-shaped structures contain SAS-6 at the proximal end of the basal body, with centrin localizing toward the growing axonemes (highlighted by tubulin staining). Basal body structures are indicated by an arrow and are enlarged in the insets. The scale bar represents 10 μm.
porates them into developing basal bodies (at least for the proteins examined here). With the development of molecular tools (27) and a complete genome sequence (http://genome.jgi-psf.org/Naegri1/Naegri1.home.html), *Naegleria gruberi* is an emerging model for basal body and centriole assembly.

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FIG. 4. The order of protein incorporation during *Naegleria* basal body assembly is SAS-6, then centrin, and finally γ-tubulin. At each time point, cells were fixed and stained with antibodies against SAS-6 (black), centrin (red), or γ-tubulin (blue), and at least 100 cells were scored for localization. The times were normalized to the point at which 50% of cells have visible flagella (65 min). Three biological replicates are indicated by circles, squares, and triangles.