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# Centrin Is Synthesized and Assembled Into Basal Bodies During *Naegleria* Differentiation

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During differentiation of *Naegleria* from vegetative amoebae to temporary flagellates, the microtubular cytoskeleton, including two basal bodies and flagella, is assembled de novo. Centrin is an integral component of these basal bodies [Levy et al., 1996, *Cell Motil. Cytoskeleton* 33: 298–323]. In many organisms, centrin appears to be a constitutive protein, but in *Naegleria* centrin gene expression occurs only during differentiation. Centrin mRNA, which has not been detected in amoebae, appears and disappears earlier in differentiation than a coordinately regulated set of differentiation-specific mRNAs encoding flagellar tubulin and calmodulin. Centrin antigen accumulates during differentiation, and then decreases in abundance as the flagellates mature and revert to amoebae. No localization of centrin has been detected in amoebae. During differentiation, centrin becomes localized to the basal bodies as soon as these structures are detected with anti-tubulin antibodies, first as a single dot and finally as two basal bodies. During reversion of flagellates to amoebae, centrin remains localized to the basal bodies for as long as they are present. When assembly of tubulin-containing structures during differentiation is prevented using oryzalin, centrin localization is prevented as well, yet inhibition of assembly does not affect accumulation of centrin antigen. Apparently in *Naegleria*, the role of centrin is primarily for a differentiation- or flagellate-specific function. The temporary presence of centrin is concurrent with the presence of centriolar basal bodies, which supports the conjecture that in *Naegleria* centrin may be needed only when these organelles are present. Cell Motil. Cytoskeleton 40:249–260, 1998. 

**Key words: centrin gene expression; centriole; amoeba-to-flagellate differentiation; oryzalin**

## INTRODUCTION

Centrin, also known as caltractin, is an acidic ≈20 kD member of the EF-hand family of calcium-binding proteins [Huang et al., 1988b]. A sufficient number of centrin genes have been cloned and sequenced to assert that centrin is a strongly conserved protein, ubiquitous among eukaryotes [Schiebel and Bornens, 1995]. Since its discovery as the major component of the contractile flagellar rootlets of the alga *Tetraselmis* [Salisbury et al., 1984], centrin has consistently been found associated with structures whose contraction is mediated by calcium ions [Salisbury, 1995]. Recently, this association was extended to the spasmoneme of *Vorticella*, long known as an organelle whose dramatic contraction is dependent on the binding of $Ca^{2+}$ [Amos, 1971] and whose major protein spasmin is immunologically related to centrin [Levy et al., 1996]. More commonly, centrin has been found in more minute structures closely associated with centrosomes [Schiebel and Bornens, 1995; Salisbury, 1995]. A recent survey of the localization of centrin in diverse structures in a wide variety of eukaryotes sup-

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ports the view that a contractile system built around centrin and originally closely associated with centrosome dynamics was recruited during evolution to produce diverse and specialized centrosome-associated structures [Levy et al., 1996].

The amoeboflagellate *Naegleria gruberi* can undergo an elective, 100-min-long, 1:1 conversion or differentiation from amoebae to flagellates [Fulton, 1977a, 1993]. The amoebae, which contain abundant actin [Sussman et al., 1984] but lack cytoplasmic microtubules [Fulton, 1977b; Walsh, 1984], feed and crawl by extending pseudopodia. In contrast, the flagellates, which do not feed or divide, have an extensive microtubule cytoskeleton [Fulton, 1977b; Walsh, 1984] and swim using flagella. The flagellates are temporary and revert back to amoebae [Fulton, 1977a,b].

During differentiation the major components of the flagellar apparatus, including the two basal bodies, their flagella, a rhizoplast, and associated cortical microtubules, are assembled de novo [Schuster, 1963; Dingle and Fulton, 1966; Fulton and Dingle, 1971; Larson and Dingle, 1981; Walsh, 1984]. Furthermore, several genes encoding protein components of these organelles are transcribed and the protein products synthesized during differentiation. For example, the α- and β-tubulin of the flagella [Kowit and Fulton, 1974a; Lai et al., 1979, 1988, 1994], the 170 kD protein of the rhizoplast [Larson and Dingle, 1981], and two calmodulins [Fulton et al., 1986, 1995], are not detected in amoebae but are synthesized during differentiation to flagellates. Interestingly, the expression of several of the differentiation-specific tubulin and calmodulin genes is coordinately regulated [Fulton et al., 1986, 1995; Mar et al., 1986].

A centrin gene from *Naegleria*, expressed in bacteria, was used to raise antibodies to centrin and these antibodies were used to detect centrin-related antigens in diverse eukaryotes [Levy et al., 1996]. Using immunofluorescence and immunoelectron microscopy we have shown that centrin is exclusively localized to the wall and lumen of the basal bodies of *Naegleria* flagellates as well as to the proximal ends of the microtubule roots that are attached to the basal bodies. In contrast to other flagellated organisms studied (e.g., *Chlamydomonas)* [Huang et al., 1988a; Salisbury et al., 1988] centrin in *Naegleria* is not associated with the flagellar root or other structures of the flagellate, nor were any centrin-containing structures detected in amoebae [Levy et al., 1996].

In order to determine whether centrin in *Naegleria* is a constitutively expressed component, i.e., present throughout the life cycle, and also how the cellular distribution of centrin changes during differentiation, we measured the abundance of centrin mRNA and protein and examined the localization of centrin during the assembly and disassembly of the tubulin-based cytoskeleton. Our results show that in *Naegleria*, centrin is expressed during a specific elective event in the life cycle of this organism, and its abundance in the cell is closely correlated with the transient presence of the centriolar basal bodies in the flagellate.

## MATERIALS AND METHODS

### Cells and Differentiation

Amoebae of *Naegleria gruberi* strain NEG [Fulton, 1970] were grown on agar medium in association with *Klebsiella pneumoniae*, differentiated synchronously to flagellates, and the rate and extent of differentiation evaluated as described previously [Fulton and Dingle, 1967]. Stationary phase cells were used for all experiments except the immunolocalization of centrin antigen in amoebae, for which exponentially growing cells were used. The concentration of cells was determined using a Coulter Counter model ZM (Coulter Electronics, Inc., Hialeah, FL), as described [Fulton, 1970].

### Antibodies

Monoclonal antibodies against *Naegleria* α-tubulin (AA-4.3) and β-tubulin (AA-12.1), described by Walsh [Walsh, 1984], were obtained from Dr. Charles Walsh (University of Pittsburgh, Pittsburgh, PA). For our studies, these two antibodies were mixed (1:1); the mixture is herein referred to as anti-tubulin antibodies. The production, affinity purification, and characterization of polyclonal antiserum 926 against *Escherichia coli*-expressed *Naegleria* centrin is described elsewhere [Levy et al., 1996]. These anti-*Naegleria* centrin antibodies have been demonstrated to specifically detect centrin antigen in a wide variety of eukaryotes [Levy et al., 1996].

### Analysis of RNA

Extraction of total *Naegleria* RNA and isolation of poly(A)$^+$ RNA were performed as previously described [Lai et al., 1979]. Denaturing (formaldehyde) agarose gel electrophoresis and Northern blotting techniques, and other general RNA handling and analysis methods were done by standard procedures [Sambrook et al., 1989]. Quantitative RNA measurements were done in triplicate as described [Lai et al., 1988]. A cDNA clone encoding *Naegleria* flagellar calmodulin [Fulton et al., 1995], and a cDNA clone encoding *Naegleria* centrin [Levy et al., 1996] were used as probes on RNA blots.

### Electrophoresis and Immunoblot Analysis of Protein Samples

Total cell protein samples were prepared from *Naegleria* by pelleting approximately $1 \times 10^6$ cells in a microfuge tube and resuspending the pellet in 1X Laemmli sample buffer [Laemmli, 1970] plus a protease inhibitor

cocktail (PMSF to 5 mM, TLCK [tosyl-L-lysine chloromethyl ketone] to 0.1 mM, TPCK [tosyl-L-phenylalanine chloromethyl ketone] to 0.1 mM, and aprotinin [Sigma A-6012] to 0.1 TIU/ml). Unless otherwise stated all reagents were obtained from Sigma (St. Louis, MO). Sodium dodecyl sulfate polyacrylamide gel electrophoresis [Laemmli, 1970] typically used 15% polyacrylamide microslab gels [Matsudaira and Burgess, 1978] run at 200V for 80 min. Proteins were transferred to Immobilon-P™ PVDF membranes (Millipore Corporation, Bedford, MA; 0.45 mm pore size) by the method of Hulen et al. [Hulen et al., 1991] using a Bio-Rad Transblot™ cell (Bio-Rad Laboratories, Richmond, CA). Immobilized proteins were detected by incubating the blots, for 1.5 h at room temperature on a shaker, with affinity-purified anti-centrin polyclonal antibodies at 1:250 in WB (Tris-buffered-saline [25 mM Tris, pH 7.4, 100 mM NaCl] containing 0.05% Tween-20) with 1% Blot-Qualified™ BSA (Promega Corp, Madison, WI) added. The blots were washed in WB for 1 h with three changes of solution, and incubated for 2 h at room temperature on a shaker with goat-anti-rabbit IgG conjugated to $^{125}$I (New England Nuclear, Boston, MA, NEX-155; 7.9 mCi/ml) at 1:200 in WB. After washing 3 × 10 min in WB, the membrane was air dried, exposed for 12–24 h to a PhosphorImager™ screen, and analyzed with a PhosphorImager 400SP (Molecular Dynamics, Sunnyvale, CA).

### Quantitation of Immunoblots

The radioactivity in each band on immunoblots was quantitated using ImageQuant™ software (v3.2; Molecular Dynamics, Sunnyvale, CA) as recommended by the manufacturer. A rectangle was drawn, copied, and placed over each signal band and over an area of the blot just above each signal area. Thus each pair of rectangles included a rectangle that covered the signal band and a rectangle covering an equal area of gel lane. The latter was used to measure the ''lane background'' in order to compensate for any small differences in the amount of material loaded per lane. The background was defined as 0.000, the software was asked to ''integrate volume'' for each rectangle, and the corrected counts for each signal band were calculated by subtracting the lane background from the corresponding raw signal. Control blots containing a dilution series of a known amount (as determined by protein assay) [Lowry et al., 1951] of purified *E. coli*-expressed *Naegleria* centrin [prepared as described in Levy et al., 1996] were used to establish the linear range for this quantitative method. The signal per band was linear from 0 to 10 ng of centrin. The amount of centrin antigen per lane measured in the experiments presented here was within this linear range (Fig. 1).

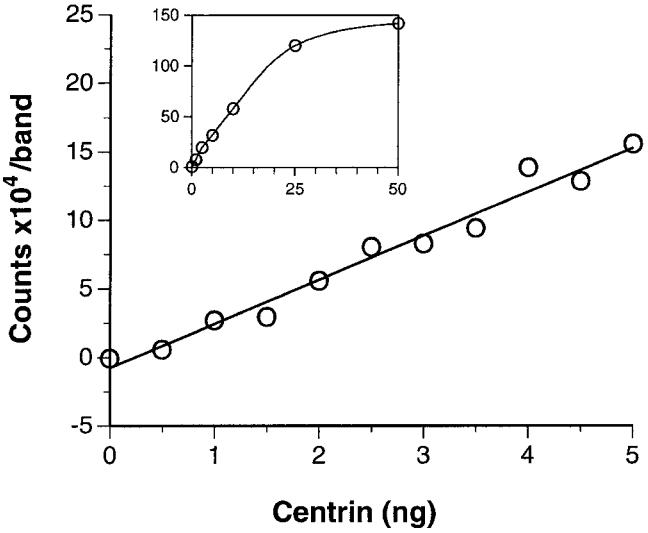


Fig. 1. Quantitation of centrin antigen. Dilution series of known amounts of purified *Naegleria* centrin expressed in *E. coli* were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis, blotted and quantitated as described in Materials and Methods. **Insert:** Linearity is lost at higher concentrations of centrin.

### Indirect Immunofluorescence Localization

Cells were fixed and permeabilized as described previously [Walsh, 1984; Trimbur and Walsh, 1992]. Multiple cell samples were handled by adhering the cells to poly-L-lysine coated multiwell slides (Carlson Scientific, Inc., Peotone, IL). Cells were simultaneously incubated with monoclonal antibodies against *Naegleria* α- and β-tubulin (each at a 1:8 dilution), and polyclonal antibodies against *Naegleria* centrin (at 1:200 dilution) for 2 h at 37°C in PBS with 0.05% Tween-20, 0.04% sodium azide, and 1% BSA added. The slides were washed in three changes of PBS-Tween for 1 h and then incubated for 1 h at 37°C with GAM-FITC and GAR-TRITC secondary antibodies (Jackson Immunoresearch Laboratories Inc., West Grove, PA) diluted to 1:100 in PBS-Tween-Azide-BSA. Following three washes as above, the slides were mounted in VectaSheild™ medium (Vector Laboratories Inc., Burlingame, CA). Specimens were viewed and photographed using a Zeiss Photomicroscope III fitted with an epi-fluorescence condenser, Zeiss 487715 and 487709 filters, and a Neofluar 100X/1.3NA objective (Carl Zeiss, Oberkochen, FRG). The Optivar was set at 2.0× and images were recorded on TMY 35 mm film (Eastman Kodak Company, Rochester, NY) processed in T-MAX developer for 10 min at 24°C. All immunofluorescence experiments included two control samples: secondary antibodies alone, and preimmune serum from the rabbit used to produce the affinity-purified anti-centrin antibodies.

## RESULTS

### Centrin mRNA Is Expressed During Differentiation to Flagellates, But Is Not Detected in Amoebae

A 0.7 kb cDNA clone encoding the full-length transcript of an expressed and sequenced centrin gene of *N. gruberi* NEG was used to estimate the abundance of centrin mRNA in RNA samples prepared from *Naegleria* amoebae and from cells at various times during differentiation. (Southern blot analysis indicates the presence of two similar centrin gene sequences in *Naegleria* that share most but not all restriction sites spanning >15 kb of genomic DNA, which we currently interpret as two alleles of a centrin gene (Levy et al., unpublished data). Only one gene or allele has been cloned and sequenced [Levy et al., 1996] (GeneBank no. U21725). No other centrin-like sequence has been detected, even using low stringency hybridization. Transcripts of either or both these similar alleles or genes would be detected on RNA blots.) Northern blot analysis showed a single-sized 0.7 kb mRNA corresponding to centrin, which is expressed during differentiation (Fig. 2A). Interestingly, centrin mRNA was not detected in amoebae (e.g., Fig. 2A, 3 min), even when blots were overexposed (data not shown).

Quantitative RNA dot blots were used to examine the change in abundance of centrin mRNA during differentiation (Fig. 2B). Centrin mRNA was not detected in amoebae, increased rapidly to a peak at around 40 min of differentiation and then decreased, declining by 100 min to a level of <5% relative to its maximum abundance. In contrast, the abundance of mRNA for flagellar calmodulin, chosen to represent a group of concurrently expressed genes including those for flagellar tubulin [Fulton et al., 1995], peaked later in differentiation, at 60 min (Fig. 2B).

### Centrin Protein Accumulates During Differentiation

To estimate the amount of centrin protein present in *Naegleria* during differentiation, SDS-PAGE samples were prepared at points during differentiation and analyzed on immunoblots probed with affinity-purified anti-centrin antibodies. These antibodies have been shown to recognize centrin-containing structures in diverse eukaryotes, and even to recognize the related yeast protein, CDC31, but not to recognize a member of the same superfamily, *Naegleria* calmodulin [Levy et al., 1996], so it is likely they recognize all centrin-like antigens and structures in *Naegleria*. Three independent experiments were done (Fig. 3A) and the abundance of centrin antigen quantitated as a percentage of the maximum level (Fig. 3B). Little centrin antigen was detected in amoebae. The relative abundance of centrin increased between 20 and 50 min of differentiation, and then remained relatively constant between 50 and 100 min. In eight separate

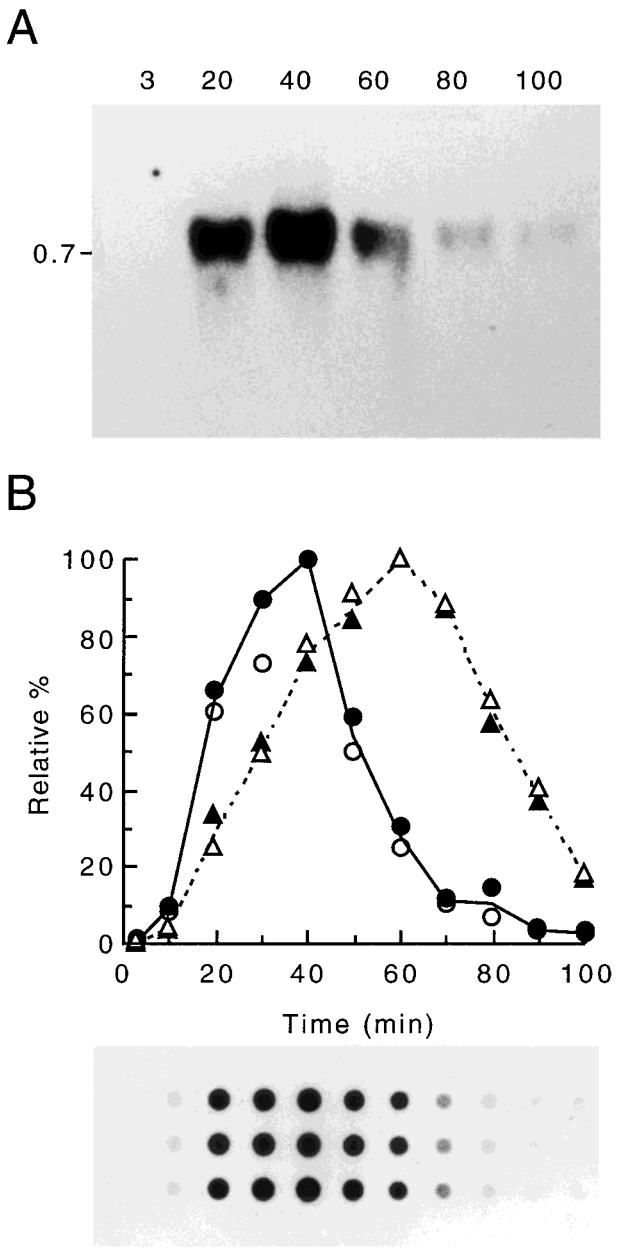


Fig. 2. The abundance of centrin mRNA rises and falls during *Naegleria* differentiation. **A:** Northern analysis of poly A+ RNA isolated from cells during differentiation revealed a single-sized ≈0.7 kb mRNA whose abundance dramatically increases and then decreases during differentiation. The time, in minutes after initiation of differentiation, is given above each lane. A 36-h exposure is shown. Even in a 7-day exposure (not shown) no mRNA was detected in amoebae (3 min). **B:** Quantitative RNA dot blot experiments for centrin mRNA (*circles*) and flagellar calmodulin (CaM-1) mRNA (*triangles*) show that abundance of mRNA for centrin peaks at about 40 min of differentiation, earlier than mRNA for flagellar calmodulin. The average abundances for centrin and for calmodulin are plotted for two separate experiments (*filled symbols*, *empty symbols*). Each RNA sample was dotted in triplicate; an example of a dot blot probed with centrin cDNA is shown below the plot.

experiments, the relative abundance of centrin in amoebae was estimated to be 8.4 ± 3.9% (mean ± s.d.) of the maximal value in flagellates. Therefore, the abundance of centrin antigen increased 12-fold during the amoeba-to-flagellate differentiation of *Naegleria*.

The average amount of centrin per flagellate was determined by including a titration of known amounts of purified *Naegleria* centrin on the same immunoblot as the samples of maximum abundance. Based on the number of cells loaded per lane, the average amount of centrin per flagellate at plateau abundance was calculated to be ≈50 ± 4 fg per cell (mean ± s.d. for the three separate differentiations). Based on the molecular mass of *Naegleria* centrin (19,610 Daltons) [Levy et al., 1996] this corresponds to $1.5 \times 10^6$ molecules of centrin per cell. A *Naegleria* flagellate has 90 pg of total cell protein [Kowit and Fulton, 1974b]; therefore, centrin comprises ≈0.06 % of the total flagellate cell protein.

### Localization of Centrin Antigen in Amoebae and During Assembly of the Flagellate Microtubular Cytoskeleton

Previous immunofluorescence localization studies detected no centrin-containing structures in *Naegleria* amoebae, but found that centrin is associated with the basal bodies of the mature flagellate [Levy et al., 1996]. Ultrastructural localization using immunogold showed that centrin is an integral component of both the wall and lumen of the basal bodies of *Naegleria* flagellates but is not found in any of the accessory structures of the flagellar apparatus, such as the inter-basal body linker or the flagellar root [Levy et al., 1996]. In the present study, we used anti-tubulin antibodies and anti-centrin antibodies to compare the localization of centrin during differentiation to that of microtubules.

An exponentially growing population of amoebae was fixed and prepared for indirect immunofluorescence localization of tubulin and centrin (Fig. 4). In many examined amoebae, including cells in interphase and in all stages of mitosis, no cellular structures, including the region at the poles of the mitotic spindles, were stained by anti-centrin antibodies.

In cells fixed at various times during differentiation (Fig. 5) distinct events in the assembly of the microtubule-based cytoskeleton were observed and related to the association of centrin with the basal bodies:

- Early in differentiation (e.g., 6 min in Fig. 5) the cells are amoeba-shaped and, with the exception of spindle microtubules in cells undergoing mitosis, no structures that stain with anti-tubulin or anti-centrin antibodies are evident.
- By 40 min after the initiation of differentiation, most cells contain a single small centrin- and tubulin-containing dot. These dots invariably are co-localized and whenever one type of dot is present, the other is also present, indicating that neither centrin nor tubulin is localized much before the other. This dot most likely represents the first detectable intermediate in the de novo formation of basal bodies in *Naegleria*.
- By 45–50 min, the cells, which are now spherical in shape, contain a pair of basal bodies that appear as a pair of short rods when stained with anti-centrin and anti-tubulin antibodies. In addition, several short tubulin-containing filaments, presumed to be the initial components of the extensive cortical microtubule cytoskeleton of the mature flagellate, are visible. By 50 min the cells have formed basal bodies with centriole structure [Fulton and Dingle, 1971]. Ten minutes later the flagella begin to be assembled at the cell surface.
- By 60 min, the cytoplasmic microtubules are organized around the proximal ends of the basal bodies, and short tubulin-containing flagellar axonemes extend from the distal ends of the basal bodies. Centrin localization clearly delineates the pair of basal bodies.
- By 70 min longer flagella are seen, the cortical cytoskeleton is more robust, and the cells begin to assume the characteristic shape of the flagellated cell.
- At 100 min after the initiation of differentiation, the development of the flagellate in terms of flagellar elongation, cell shape change, and assembly of organelles of the flagellar apparatus is complete. In contrast to tubulin, centrin localization remains restricted to the basal bodies throughout assembly of the microtubule cytoskeleton and no other structures of the developing or mature flagellate stained with anti-centrin antibodies.

This description of the localization of tubulin during assembly of the flagellate cytoskeleton is in agreement with the results of Walsh for *Naegleria* strain NB-1 [Walsh, 1984], except that study did not describe early stages of basal body formation.

In several studied organisms [Salisbury et al., 1984, 1988; McFadden et al., 1987; Baron et al., 1994] manipulation of the concentration of $Ca^{2+}$ during fixation for microscopy results in striking differences in the appearance of centrin-containing structures. Experiments in which *Naegleria* flagellates were permeabilized and fixed in the presence or absence of $Ca^{2+}$ (i.e., in the presence of 2 mM $CaCl_2$ or 2 mM EGTA) did not reveal any discernible difference in centrin or tubulin staining (data

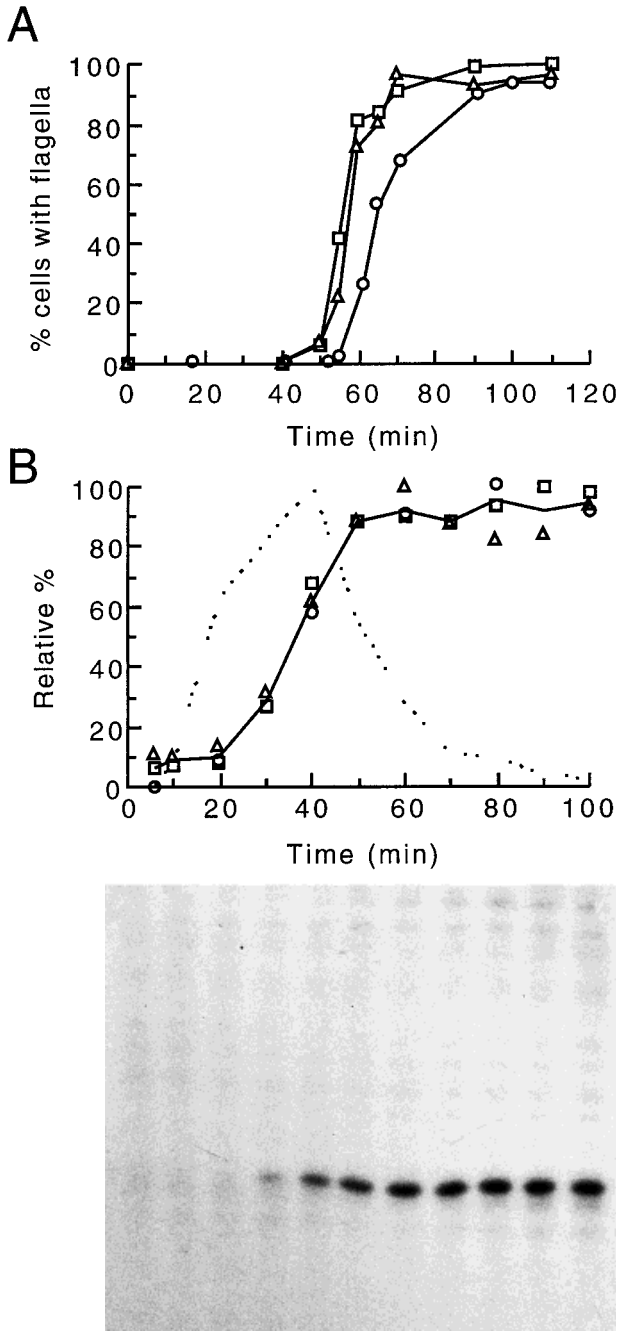


Fig. 3. The abundance of centrin protein increases during *Naegleria* differentiation. **A:** Three independent differentiation experiments were done. Cells were fixed at the indicated times in Lugol's iodine, and scored for the presence of visible flagella. The $T_{50}$ for the appearance of flagella was 64 min for Experiment 1 (*open circle*), 56 min for Experiment 2 (*open square*), and 58 min for Experiment 3 (*open triangle*). **B:** Protein samples were prepared at various times during each differentiation, blotted, stained with anti-centrin antibodies, and quantitated as described in Materials and Methods. The abundance (percent of the maximum level) of centrin protein for each experiment is plotted (symbols as above), along with the average of the three experiments (—— ). For comparison, the abundance of centrin mRNA during differentiation is displayed (- - - - ; Fig. 2). An example of one of the immunoblots (Experiment 2) is shown below the plot, with the times of sampling indicated on the x-axis of part B. Total cell protein from about 37,000 cells was loaded per lane.

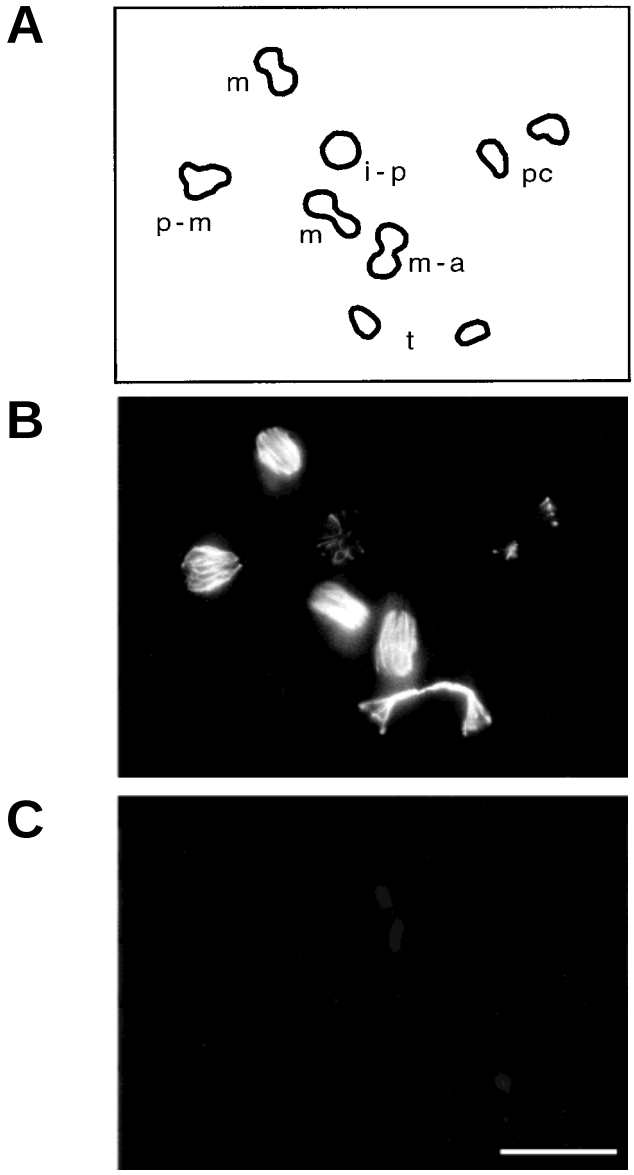


Fig. 4. In amoebae tubulin is localized to the mitotic spindle but no centrin is detected. An exponentially growing population of amoebae was fixed and stained with anti-tubulin and anti-centrin antibodies. **A:** A tracing (from a phase-contrast micrograph) of the nuclei of seven cells at various stages of mitosis. The nuclei are labeled according to their mitotic stage based on previous studies using Feulgen stain [Fulton, 1970] and indirect immunofluorescence localization of α- and β-tubulin [Walsh, 1984]: i-p, interphase-prophase; p-m, prophase-metaphase; m, metaphase; m-a, metaphase-anaphase; t, telophase; pc, post-cytokinesis. The nuclei of interphase cells present in the same field do not contain any microtubules and are not labeled. **B:** The identical field of cells showing the localization of α- and β-tubulin staining in the spindles of the mitotic cells. **C:** The identical field of cells showing the absence of centrin localization in mitotic or interphase amoebae. Scale bar = 10 μm.

not shown). Neither the position of the basal bodies with respect to the nucleus, nor the orientation of the basal bodies with respect to each other, nor any other feature detected at the resolution of the light microscope was influenced by the presence or absence of $Ca^{2+}$.

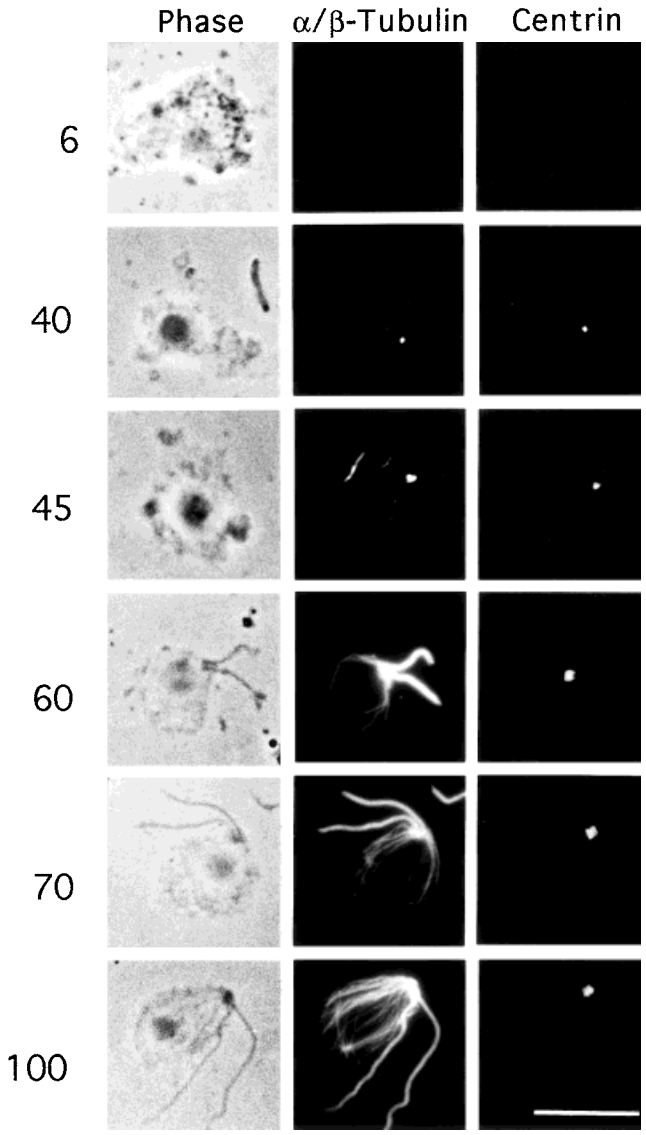


Fig. 5. Assembly of the microtubule cytoskeleton during the differentiation of *Naegleria* flagellates and restriction of centrin to the basal bodies. Cells were fixed at the indicated times in minutes after initiation of differentiation and stained with anti-tubulin and anti-centrin antibodies. The cells are shown under phase contrast optics, with tubulin staining, and with centrin staining as indicated. The scale bar = 10 μm.

**TABLE I. Maximal Abundance of Centrin Antigen in Control and Oryzalin-Treated Cells***

| Experiment | Treatment | Centrin/cell (fg) | Percent difference |
|---|---|---|---|
| 1 | Control | 44.7 | −5.3 |
| | Oryzalin | 47.2 | |
| 2 | Control | 54.5 | +8.8 |
| | Oryzalin | 49.7 | |
| 3 | Control | 51.3 | +8.8 |
| | Oryzalin | 46.8 | |

*Each experiment consisted of two separate pairs of differentiations: one a control and the other with $5 \times 10^{-7}$ M oryzalin added at 6 min after initiation of differentiation.

### Inhibition of Tubulin Polymerization Prevents Centrin Localization But Does Not Affect Centrin Synthesis During Differentiation

Because centrin is intimately associated with, and restricted to, the basal bodies of the flagellate, and its assembly is concurrent with the assembly of tubulin, we were interested to determine whether centrin synthesis and localization are dependent on microtubule assembly. The herbicide oryzalin binds to plant tubulin in vitro and inhibits mitosis in plants [Morejohn et al., 1987], but has no effect on mitosis in animal cells and does not bind to mammalian tubulins [Hugdahl and Morejohn, 1993]. Oryzalin prevents assembly and causes disassembly of microtubules in *Naegleria* flagellates (Lai et al., unpublished data). When *Naegleria* amoebae were differentiated in the presence of oryzalin (0.5 μM), the cells did not produce flagella and did not lose their amoeboid shape and motility (Fig. 6A). A small fraction of the cells transiently lost their amoeboid shape (round up) at the appropriate time, but then quickly went back to amoebae. However, the presence of oryzalin had little if any effect on the kinetics of accumulation of centrin antigen during differentiation (Fig. 6B), or on the average amount of centrin antigen per cell that accumulated (Table I). Similarly, oryzalin does not affect the expression and accumulation of α- and β-tubulin mRNA and protein during differentiation (Lai et al., unpublished data). Cells incubated in the presence of oryzalin were fixed at 100 min after the induction of differentiation and stained with antibodies against centrin and tubulin (Fig. 6C). Oryzalin-treated cells did not form flagellar axonemes, centrin- or tubulin-containing basal bodies, or cortical microtubules but did contain a variable number of small spots which stained with anti-tubulin but not with anti-centrin antibodies. Tubulin spots were never observed in control populations of cells. The prevention of both basal body assembly and centrin localization in oryzalin-treated cells suggests that the localization of centrin is dependent on the assembly of some differentiation-specific cellular structure, quite possibly the tubulin-containing basal bodies themselves.

### Localization of Centrin During Disassembly of the Flagellate Cytoskeleton

*Naegleria* flagellates are temporary and non-feeding, and under the conditions used here revert back to vegetative amoebae within an hour or two after becoming mature flagellates. The expression pattern of centrin mRNA during differentiation and the localization of centrin protein in flagellates suggests that centrin may be required only for a flagellate-specific function. During reversion, the flagella are retracted into the cell body with dramatic rapidity, so it is of interest to determine whether the distribution of centrin changes during this potentially

contractile process. Therefore, immunolocalization and immunoblot studies were conducted to determine the fate of centrin protein during reversion.

Reversion of *Naegleria* flagellates to amoebae has not been extensively described in the literature and a thorough description of this process is beyond the scope of this paper. Observation of live cells indicates that reversion of individual cells appears to be a stochastic process in which some cells revert within minutes of becoming flagellates while others last much longer. Consequently, compared to differentiation, reversion of the population is less synchronous and less reproducible from experiment to experiment. Cells undergoing reversion were observed to lose their flagellate shape and resume amoeboid motility within a few seconds [Fulton, 1977b]. The cells crawl along the substrate and drag their non-functioning flagella behind them for some minutes before suddenly retracting their flagella (Dingle, Levy et al., unpublished observations). The ephemeral microtubule cytoskeleton of *Naegleria* is disassembled during reversion [Fulton, 1970, p. 399].

In order to examine any changes in the tubulin cytoskeleton or redistribution of centrin antigen during reversion, cells were fixed and prepared for indirect immunofluorescence localization of tubulin and centrin at various times after differentiation was complete. Figure 7 shows representative cells at various stages in the process of disassembly of the cortical cytoskeleton, flagella, and basal bodies during reversion. Prior to reversion (stage 0 in Fig. 7) the flagellates have a streamlined cell body, a tubulin-containing cytoskeleton, and centrin-containing basal bodies. Five stages were identified in the continuous process of reversion:

Stage 1. In the earliest stage of reversion, the cells lose their flagellate-shape but the flagella remain external. The cortical microtubules are no longer straight and often are excluded from the amoeboid end of the cell (i.e., the end with the leading pseudopod). Centrin remains associated with the basal bodies.

Stage 2. The flagella have been internalized but the flagellar apparatus remains intact with flagella, basal bodies, and cortical microtubules attached as a unit. The cortical microtubules are fewer and shorter. There is no change in centrin distribution or subjective change in intensity of centrin

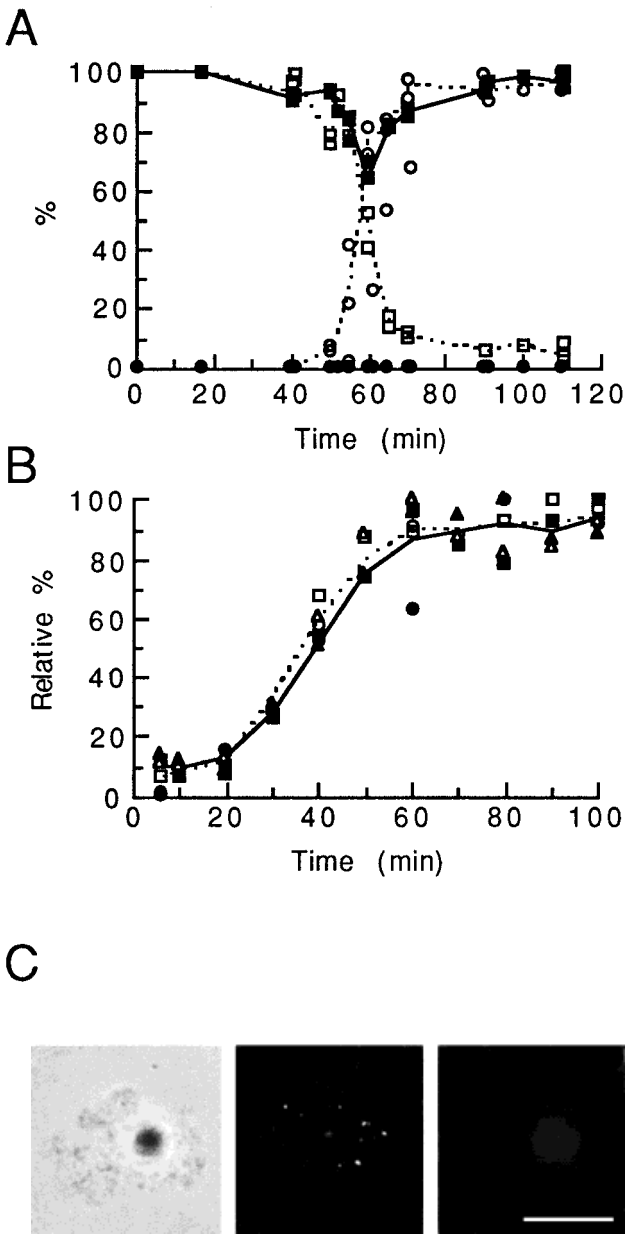


Fig. 6. The effect of oryzalin on differentiation to flagellates, expression of centrin antigen, and localization of centrin and tubulin during differentiation. **A:** Cells differentiated in the presence (*filled symbols*; *solid line*) or absence (*open symbols*; *dotted line*) of $5 \times 10^{-7}$M oryzalin were fixed in Lugol's iodine at various times after induction of differentiation. For each sample 100 cells were scored for presence of visible flagella (*circles*), and for amoeboid shape (*squares*). Data represents the average of three separate experiments. In contrast to untreated cells, cells incubated under differentiation conditions in the presence of oryzalin did not form flagella and retained their amoeboid shape, except for a transient rounding up of some of the cells at the time most of the control cells rounded up. **B:** Protein samples were prepared at various times from cells differentiated in the presence (*filled symbols*) or absence (*open symbols*) of $5 \times 10^{-7}$ M oryzalin, blotted, stained with anti-centrin antibodies, and quantitated as described in Materials and Methods. The abundance (percent of the maximum level) of centrin protein for each experiment is plotted along with the average of the three experiments (control, - - - - ; oryzalin-treated, ——). **C:** Oryzalin-treated cells were fixed and prepared for indirect immunofluorescence localization of tubulin and centrin at 100 min of differentiation. A representative cell is shown under phase contrast optics, with tubulin staining, and with centrin staining. The scale bar = 10 μm.

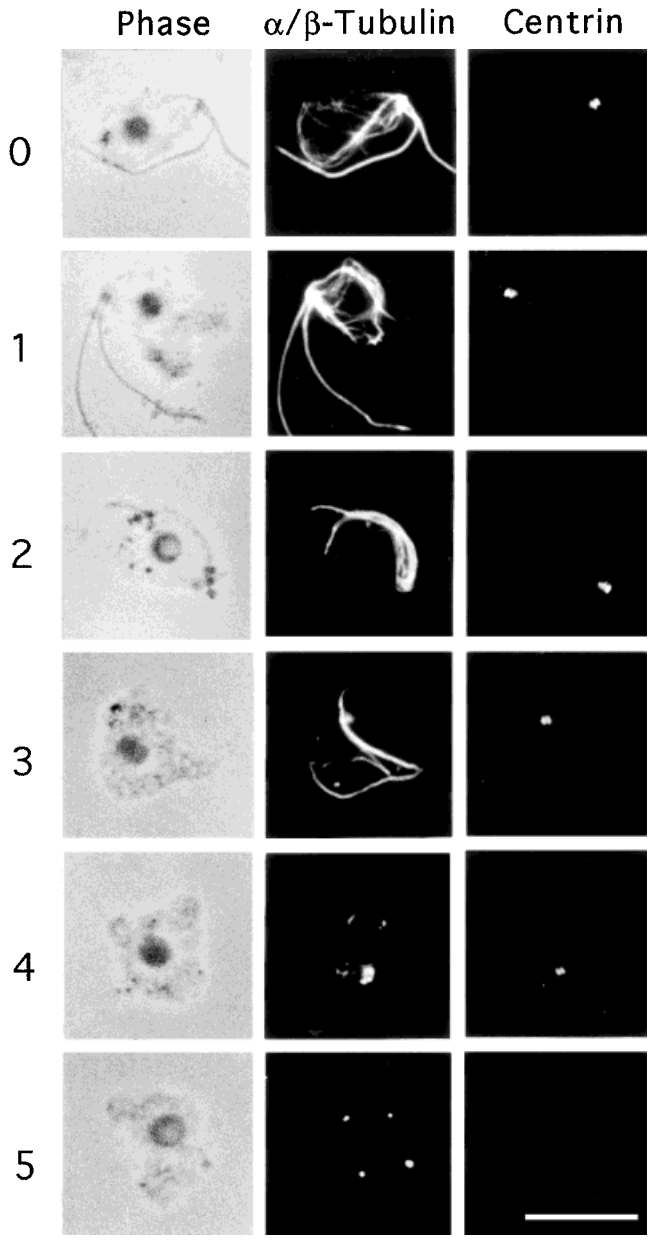


Fig. 7. Disassembly of the microtubule cytoskeleton during reversion of *Naegleria* flagellates and persistence of centrin in the basal bodies until they are disassembled. A heterogeneous population of reverting cells was fixed and stained with anti-tubulin and anti-centrin antibodies. Cells representative of each of the stages of reversion (indicated on the left) are shown under phase contrast optics, with tubulin staining, and with centrin staining as indicated. The scale bar = 10 μm.

staining at the basal bodies. Cells at this stage were rare, suggesting that this stage is short-lived.

Stage 3. The individual flagella and any remaining cortical microtubules are detached from the basal body pair and are separate in the cytoplasm. The cortical microtubules are largely gone, and tubulin-containing spots, similar to those observed in oryzalin-treated cells, first appear. In some cells the flagella are frayed at their ends into finer filaments, suggesting that the microtubules of the axoneme are beginning to laterally disassociate. The basal bodies persist as a pair and centrin remains localized to them.

Stage 4. All tubulin-containing structures of the flagellate except the basal bodies are gone and the cells contain a greater number of tubulin-containing spots. The basal bodies appear normal or slightly diffuse by tubulin staining but centrin localization appears normal. Like stage 2, cells at this stage are rare, suggesting that cells pass through this stage rapidly.

Stage 5. The cells are indistinguishable from interphase amoebae except for a small number (usually 0 to 6) of tubulin-containing spots that eventually disappear. The basal bodies are no longer present as defined by tubulin or centrin localization.

**TABLE II. Stages in the Reversion of Flagellates to Amoebae***

| Time (min) | Stage 1 | 2 | 3 | 4 | 5 | Reverting |
|---|---|---|---|---|---|---|
| 120 | 78 | 0 | 10 | 4 | 8 | 18 |
| 150 | 80 | 8 | 4 | 8 | 0 | 46 |
| 180 | 21 | 12 | 31 | 6 | 27 | 69 |
| 210 | 2 | 4 | 10 | 12 | 72 | 82 |
| 240 | 1 | 3 | 1 | 4 | 91 | 100 |

*Following differentiation, cells were fixed at the times indicated and double-stained for indirect immunofluorescence localization of tubulin and centrin. For each time point (given in minutes after induction of differentiation), 100 reverting cells were scored into one of the five stages of reversion referred to in the text. ''Reverting'' cells were defined as cells that did not have the characteristic flagellate-shape. For each time point, the percentage of cells at each stage is indicated along with the percentage of reverting cells in the cell population. Despite the unavoidable asynchrony of the population, a progression to later stages of reversion over time is clearly evident.

Despite the relatively asynchronous occurrence of reversion, this sequence of stages during reversion was verified by examination of cells fixed at various times after differentiation was complete and scoring them into one of these stages. The results of these counts are shown in Table II. The proportion of cells in different stages changed over time in a manner consistent with the order in which the stages were logically thought to occur. Centrin distribution did not detectably change upon internalization of the flagella, or during any other stage of reversion. Centrin remained localized at the basal bodies until they were finally disassembled.

### Abundance of Centrin Protein Decreases Following Differentiation

At 240 min after the initiation of differentiation (about 120 min after mature flagellates formed) less than 10% of the cells contained basal bodies as defined by tubulin and centrin immunolocalization (Table II). In order to determine whether the disassembled centrin persists in reverted cells, and if so, for how long, the amount of centrin antigen in cells at various times after differentiation was measured. Three differentiation experiments were done and the population of cells allowed to revert (Fig. 8A). Cells with flagella persisted for 1 to 3 hours after they formed. Total cell protein samples were prepared at various times up to 5 hours from the beginning of differentiation. Immunoblots of these samples were probed with affinity-purified anti-centrin antibodies and the amount of full-size centrin antigen quantitated (Fig. 8B). Centrin protein remained detectable throughout reversion but by 300 min after induction of differentiation the abundance of centrin was only 30 to 60% of the maximal amount present in flagellates. A small amount of lower molecular weight centrin antigen, presumably partially degraded centrin, was detected (Fig. 8C). A transient small increase in centrin abundance was reproducibly seen at about 180 min after induction (Fig. 8B); this increase may be due to a small fraction of the cells that differentiate a second time starting at about 120 min. Re-differentiation has been documented in strain NB-1 [Fulton and Dingle, 1967], and occurs to a lesser extent in strain NEG [Fulton, 1970]. These results show that in reverting cells the abundance of centrin antigen decreases soon after reaching the maximal amount, with a half life of roughly 180 minutes.

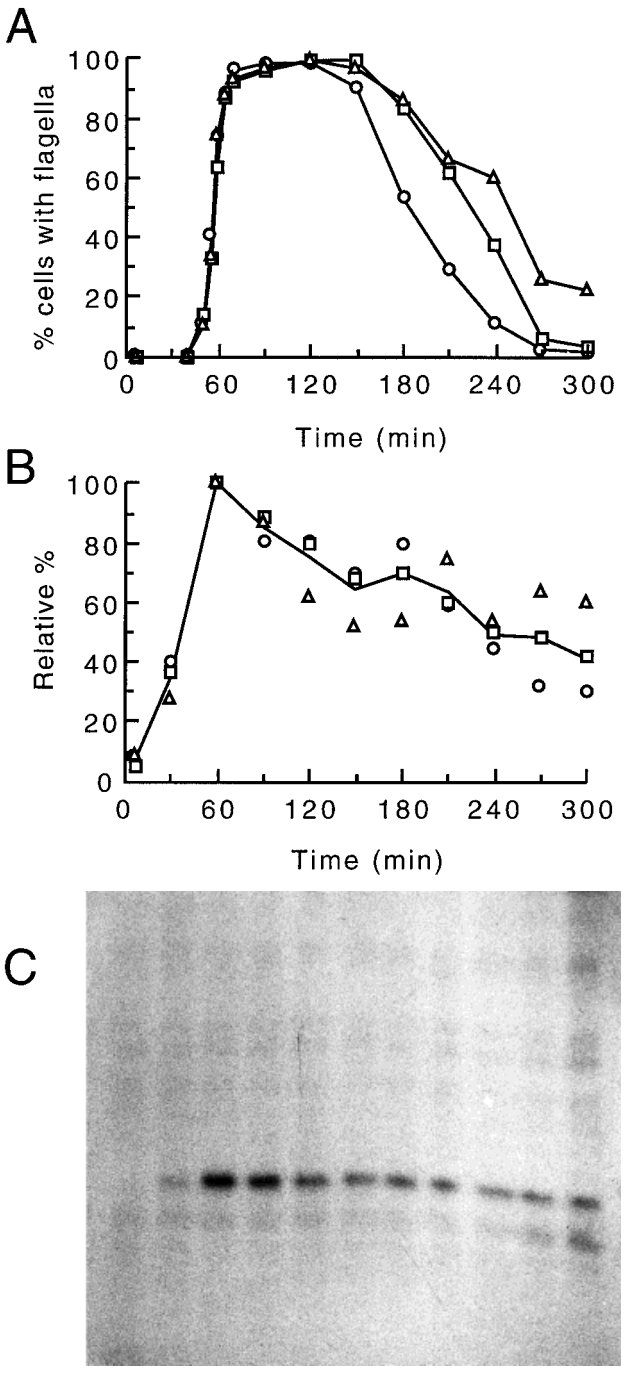


Fig. 8. The abundance of centrin antigen decreases soon after mature flagellates are formed. **A:** Three independent differentiation experiments were done (*open symbols*). Cells were fixed at various times in Lugol's iodine, and scored for the presence or absence of visible flagella. The $T_{50}$ for the appearance of flagella was 57 min for all three experiments. Note that the loss of flagella during reversion is less synchronous and less reproducible than the appearance of flagella during differentiation. **B:** Protein samples were prepared at various times during differentiation, blotted, stained with anti-centrin antibodies, and quantitated as described in Materials and Methods. The abundance (percent of the maximum level) of centrin protein for each experiment is plotted (*symbols as above*), along with the average of the three experiments (——). **C:** An example of one of the immunoblots (Experiment 2, *open square*) is shown below the plot, with the times of sampling indicated on the x-axis of part B. Total cell protein from about 23,500 cells was loaded per lane. A small amount of low molecular weight centrin antigen, presumably partially degraded centrin, was reproducibly observed at 240 and 300 min after the initiation of differentiation.

## DISCUSSION

Amoeboflagellates exhibit dual modes of existence: the amoebae feed, divide, and crawl using pseudopodia, and the flagellates, which neither feed nor divide, swim using flagella. *Naegleria* is notable for its centriole-less amoebae and de novo formation of basal bodies [Fulton and Dingle, 1971]. We present results indicating that in *Naegleria*, centrin is differentially expressed during the assembly of the tubulin-based cytoskeleton of the flagellate. Furthermore, centrin expression is on an earlier time course than the coordinated expression observed for flagellar calmodulin and the α-tubulin and β-tubulin gene families that are expressed during differentiation [Fulton

et al., 1995]. Centrin mRNA abundance peaks about 20 min earlier than flagellar calmodulin and tubulin mRNAs; the centrin gene is the earliest gene so far identified in the programmed expression of genes during differentiation. The abundance of centrin antigen increased beginning about 20 min after the induction of differentiation until by 50 min, at which time basal body assembly is largely complete, the cells have accumulated the maximum amount of centrin. Upon reversion of the flagellates back to amoebae and subsequent disassembly of the microtubule-based cytoskeleton, including the basal bodies, the abundance of centrin antigen decreases. The differentiation-specific expression of the centrin gene(s) strongly suggests that centrin in *Naegleria* is used for some differentiation- or flagellate-specific function, which is not required in the centriole-less amoeba.

Major changes in centrin gene expression have not been documented in any other organism and centrin is generally considered to be constitutively present. Consistent with our demonstration of the close association of centrin antigen with the basal bodies of *Naegleria* flagellates and with the centrioles of diverse eukaryotes [Levy et al., 1996], it is intriguing to speculate that changes in centrin expression have not been seen in other studied organisms, such as *Chlamydomonas* and mammalian cells in culture, because the basal bodies or centrosomes are present throughout the life cycle of these cells.

Despite the lack of detectable centrin mRNA in amoebae, amoebae contain ≈8% of the centrin antigen of flagellates. It is recognized that some of the antigen present in amoebae may be non-specific ''noise'' (see blot, Fig. 3). One possibility, perhaps the most likely, is that amoebae have a protein related to centrin that is encoded by a gene too divergent to be detected using the centrin DNA probe and low-stringency hybridization (our unpublished data), but which shares antigenic determinants with centrin. Another possibility is that amoebae have very stable centrin synthesized under the direction of minimal, undetected mRNA encoded by the same gene that is expressed during differentiation, and that the amount of protein increases 12-fold during differentiation. A third possibility is that 8% of the amoebae contain the amount of centrin found in flagellates; we consider this unlikely because of the homogeneity of the clonal cell population. The uncertainty about the nature of the ≈8% of cross-reacting antigen in amoebae leaves open the questions of whether the centriole-less amoebae contain bona fide centrin or a related protein and whether centrin is encoded by an essential gene in *Naegleria*.

In addition to the differential expression of the centrin gene(s) and accumulation of centrin protein during differentiation, we document here the close association of centrin with the basal bodies during the assembly and disassembly of these organelles. The failure of centrin to be localized when tubulin polymerization is inhibited by oryzalin, or to remain localized after basal body disassembly during reversion, suggests that centrin localization is dependent on assembly of other structural elements. This could mean that centrin and tubulin interact directly or indirectly with each other, or simply that if tubulin, the major component of the basal bodies, is prevented from forming the ''core'' on which the other basal body components assemble, then the other components including centrin do not become localized. Significantly, these results imply that centrin does not independently interact with some non-tubulin-containing ''basal body precursor,'' which might be present in the cells prior to the recruitment of tubulin to the nascent basal bodies.

Experiments in which cells were fixed in the presence and absence of $Ca^{2+}$ failed to reveal any discernible difference in centrin localization, or in basal body position or orientation within the cell. This is in contrast to what has been observed with several unicellular algae, most notably *Spermatozopsis*, in which the distal fiber, a band-like connector that links the two basal bodies and which contains centrin antigen, markedly changes its shape in response to elevated calcium concentrations and thereby changes the relative orientation of the basal bodies [McFadden et al., 1987]. Furthermore, centrin distribution did not detectably change upon internalization of the flagella during reversion, and the ultrastructural localization of centrin in *Naegleria* found no centrin associated with obvious filamentous structures [Levy et al., 1996]. Therefore, the function of centrin in the *Naegleria* flagellate appears to be not in gross contractile motility but in some unknown, more subtle, functioning of the basal bodies.

The ultrastructural localization of centrin to the basal bodies of the *Naegleria* flagellate revealed that centrin is found in both the wall and lumen of the basal bodies, but that it is asymmetrically distributed within these structures [Levy et al., 1996]. Specifically, there is a concentration of centrin near the point of attachment of the microtubule rootlets. These structures, which appear to be composed of microtubules, are the main trunks of the extensive cortical microtubule cytoskeleton of the flagellate. Therefore, centrin is located where it could conceivably be involved in the attachment, detachment, or positioning of the microtubule rootlets, and therefore cortical cytoskeleton, in relation to the wall of the basal bodies. Such activity could be important both during the assembly of the various components during differentiation as well as during reversion when the axonemes and cortical microtubules are severed from the basal bodies. *Naegleria* differentiation affords unique opportunities for studies addressing the regulation of centrin synthesis and assembly. At this stage of our knowledge, it is especially provocative that centrin gene expression and the synthesis and assembly of centrin are tightly concurrent with the

temporary assembling and functioning of the centriolar basal bodies during *Naegleria* differentiation.

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