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A novel structural component of the *Dictyostelium* centrosome

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SUMMARY

The microtubule-organizing center of *D. discoideum* is a nucleus-associated body (NAB) that consists of a multilayered, box-shaped core embedded in an amorphous corona from which the microtubules emerge. The composition of the NAB is still largely unresolved. Here we have examined a high molecular mass component of the NAB which was identified by a monoclonal antibody raised against isolated nucleus/NAB complexes. This antibody recognized a 350 kDa component which is immunologically related to the *D. discoideum* heavy chain of myosin II. The 350 kDa antigen was localized only at the NAB in interphase cells, while in mitotic cells it may also be found in the vicinity of the NAB as well as in association with the mitotic spindle. Immunogold labeling experiments showed that the protein is part of the NAB corona. This association was not destroyed by treatment with 2 M urea or 0.6 M KCl. The 350 kDa antigen was part of the thiabendazole-induced cytoplasmic microtubule-organizing centers. A direct role in the polymerization of tubulin could not be determined in an in vitro microtubule nucleation assay, whereas antibody electroporation of live cells appeared to interfere with the generation of a normal microtubule system in a subset of cells. Our observations suggest that the 350 kDa antigen is a structural component of the NAB corona which could be involved in its stabilization.

Key words: Centrosome, *Dictyostelium discoideum*, NAB, MTOC

INTRODUCTION

In most animal, many fungal, and some plant cells the centrosome is the major microtubule-organizing center (for a review see Kalnins, 1992). Centrosomes determine the polarity, number, structural fidelity and initial orientation of a cell's microtubules. Centrosomal morphologies may differ widely in different organisms and cell types. In most animal cells, they consist of a pair of centrioles surrounded by an amorphous cloud of electron-dense material, the pericentriolar matrix (PCM). The latter is responsible for microtubule organization (Gould and Borisy, 1977), whereas removal of the centrioles dissociates cell growth from cell division (Maniotis and Schliwa, 1991). In fungi and some protists, the centrosome is a nucleus-associated body of variable size, shape, and density that lacks centrioles (Heath, 1981) and, in some cell types, may undergo complex structural rearrangements during the cell cycle (e.g. Byers and Goetsch, 1975).

The compostion of a given centrosome is largely unknown, but likely to be complex (Kalt and Schliwa, 1993). Recent molecular, genetic, biochemical, and immunological studies have identified a variety of potential centrosomal components, three of which, to-date, are candidates for building blocks of all centrosomes: γ-tubulin, centrin, and pericentrin. γ-Tubulin (Oakley and Oakley, 1989) has been found in association with a wide variety of centrosomes (Joshi, 1994) where it comprises a ring-shaped structure responsible for microtubule nucleation (Joshi et al., 1992; Zheng et al., 1995; Moritz et al., 1995). Centrin (Salisbury et al., 1984) or centrin-like proteins are small, calcium-binding molecules that localize to centrosomal structures in various species (Lee and Huang, 1993; Errabolou et al., 1994; Schiebel and Bornens, 1995). Their precise function is still unknown, though they undergo calcium-induced, ATP-independent contraction (Baron and Salisbury, 1992). Pericentrin (Doxsey et al., 1994) is a molecule of unknown function that is recognized in a wide variety of cells and organisms by the human centrosomal autoantibody 5051 (Calarco-Gillam et al., 1983) and, therefore, is likely to be evolutionarily conserved. There are many other proteins or antigens that are localized at the centrosome (Kalt and Schliwa, 1993), but in most cases it is too early to tell whether they represent universal centrosomal constituents.

To identify and characterize components of the centrosome, we have chosen the slime mold *Dictyostelium discoideum* as a model system. The *D. discoideum* centrosome, also called nucleus-associated body (NAB; Roos, 1975), is a box-shaped, layered structure surrounded by an amorphous corona from which microtubules emerge (Omura and Fukui, 1985; Kuriyama et al., 1982). The simplified morphology of this organelle in a cell type that otherwise exhibits a complex cytoskeleton and behavior reminiscent of higher eukaryotic cells makes *D. discoideum* an excellent model system for the characterization of centrosomal components and their function.

Here we report on the identification and initial characterization of a novel component of the *D. discoideum* centrosome (NAB). This high molecular mass protein is localized to the corona, a peripheral layer involved in microtubule nucleation, and presumably plays a structural or stabilizing role.

MATERIALS AND METHODS

Cell culture

The axenic AX2 strain was grown in AX medium (14.3 g peptone, 7.15 g yeast extract, 18 g maltose, 3.5 mM Na_2HPO_4, 3.5 mM KH_2PO_4, pH 6.7; Claviez et al., 1982) on a rotary shaker at 21°C, 150 rpm. The MHC-null mutant strain HS2205 was grown in Petri dishes using AX medium supplemented with 10 mg/ml G418 (Sigma) (Manstein et al., 1989).

Generation of antibodies

Nucleus/NAB complexes for the immunization of mice were prepared by the lysis of exponentially growing AX2 cells, based on methods by Bornens et al. (1987), Kuriyama et al. (1982), and Pederson (1977). 10^8 cells per ml lysis buffer I (10% sucrose, 1 mM Tris-HCl, pH 8.0, 0.5% Triton X-100, 2 mM $MgCl_2$, 1 mM PMSF, 1 mg/ml pepstatin A, 10 mg/ml leupeptin) were lysed at 4°C with vortexing for 1 minute. Lysis was monitored microscopically. 50 ml of the released nucleus/NAB complexes were spun through a 1 ml sucrose cushion (30% sucrose, 2 mM $MgCl_2$, 1 mM PMSF, 1 mg/ml pepstatin A, 10 mg/ml leupeptin in Soerensen phosphate buffer, pH 6.0, 14.6 mM KH_2PO_4, 2 mM Na_2HPO_4; Malchow et al., 1972) at 4°C, 2,000 ***g*** for 10 minutes. Each 1 ml pellet was resuspended in 5 ml lysis buffer I without detergent and sedimented through a 1 ml sucrose cushion (2,000 ***g***, 10 minutes, 4°C). This step was repeated twice until the supernatant became clear. The nucleus/NAB complexes were stored in the sucrose cushion at −80°C.

For the immunization of Balb/c mice 200 mg nucleus/NAB complexes, treated with 0.1% SDS were injected. Hybridoma culture supernatants were tested in immunofluorescence and western blots of isolated nucleus/NAB complexes for the production of NAB antibodies. The NAB350 hybridoma cell line secreted an IgG. The antibody was purified from culture medium by ammonium sulphate precipitation and chromatography on Protein A-Sepharose (Sigma).

Immunofluorescence microscopy

Cells suspended in medium were allowed to settle on a coverslip for 10 minutes. The cells were fixed for 5 minutes using 3.7% formaldehyde, 0.05% glutaraldehyde, 0.5% Triton X-100 in 50% PHEM (PHEM, pH 6.9, 60 mM Pipes, 25 mM Hepes, 10 mM EGTA, 2 mM $MgCl_2$; Schliwa et al., 1982). After three washes with Soerensen phosphate buffer the cells or complexes were incubated in sequence at room temperature for 30 minutes with the primary antibody, followed by the corresponding secondary antibody, both diluted in PBS containing 1% BSA and 0.1% Na-azide. Each incubation was followed by three washes. This sequence was repeated for double immunofluorescence labelling. Nuclei were stained with DAPI (Sigma). Slides were mounted in Gelvatol.

To better visualize the distribution of myosin II, cells were treated by the agar-overlay method (Yumura et al., 1984) prior to fixation. These cells were then fixed according to the method of Fukui et al. (1987) in 99% methanol, 1% glutaraldehyde at −10°C for 10 minutes. The primary antibodies used were NAB350, a mouse-derived monoclonal NAB antibody, obtained as undiluted culture supernatant or affinity-purified antibody (working dilution 1:100); YL 1/2 (Kilmartin et al., 1982), a rat-derived monoclonal *Saccharomyces* uvarum α-tubulin antibody (working dilution 1:1,200) and mAb96 (Claviez et al., 1982; Pagh and Gerisch, 1986), a mouse-derived *D. discoideum* MHC antibody (working dilution 1:100). The secondary antibodies were a Cy3-labeled goat anti-mouse IgG (Dianova, working dilution 1:1,000), a fluorescein-labeled goat anti-rat IgG (Cappell, West Chester, PA, working dilution 1:40) and a fluorescein-labeled goat anti-mouse IgG (Dianova, working dilution 1:40).

Microscopic observation was done using a Zeiss Axiophot microscope equipped with DAPI, rhodamine, and fluorescein channels. Images were taken on Agfa T-Max 100 or T-Max 400 films.

Electrophoresis and immunoblotting

Cells or isolated nucleus/NAB complexes were solubilized in sample buffer according to the method of Laemmli (1970). Proteins were separated on a discontinous 4.5% polyacrylamide gel following the methods of Garfin (1990; based on the method of Laemmli, 1970), and then electrophoretically transferred to nitrocellulose filters for 1 hour at 400 mA according to the method of Towbin et al. (1979). Proteins were visualized on the filters with 0.25% Ponceau S (Sigma) in 40% methanol and 15% acetic acid. The filters were blocked using 5% non-fat dry milk in TBST (20 mM Tris-HCl, pH 7.4, 150 mM NaCl; Sambrook et al., 1989; and 0.05% Tween-20). After three washes in TBST the filters were incubated in primary antibody, diluted in TBST, at room temperature for 1 hour. After three washes the filters were incubated in secondary antibody (horseradish peroxidase-conjugated goat anti-mouse IgG, Bio-Rad, diluted in TBST, 1:2,000) at room temperature for 1 hour. The reaction was visualized using enhanced chemoluminescence (ECL, Amersham).

Immunogold electron microscopy

Nucleus/NAB complexes, either untreated or carefully suspended in 2 M urea or 0.6 M KCl at 4°C for 5 minutes, were fixed and washed according to the protocol also employed for immunofluorescence microscopy. After an incubation in NAB350 for 45 minutes at room temperature, the complexes were washed three times in PBS, pH 8.2, containing 1% BSA. The complexes were incubated overnight at room temperature with a goat anti-mouse IgG gold antibody, diluted in PBS/BSA. After three washes in PBS/BSA, the complexes were fixed according to the method of Karnovsky (1965) in 1% formaldehyde, 3% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, for 1.5 hours, followed by 1% osmium tetroxide for 1 hour with a washing step in 0.1 M cacodylate buffer between the two fixations. After dehydration with ethanol the complexes were embedded by inversing Epon/Araldite-filled capsules on the coverslip. After polymerization for 24 hours at 60°C the coverslip was removed through insertion into liquid nitrogen. Sections were cut with a Reichert Ultracut, collected on grids and contrasted with 0.5% Mg-uranyl acetate for 20 minutes followed by 10 minutes in lead citrate (1.33 g lead nitrate, 1.76 g Na-citrate in 30 ml water; Reynolds, 1962). Sections were examined in a JEM-1200 EXII Electron Microscope (JEOL, Tokyo, Japan).

Induction of cytoplasmic MTOCs

Cytoplasmic MTOCs were induced by the incubation of AX2 cells with 10^{-4} M thiabendazole (Kitanishi-Yumura et al., 1985) for 24 hours. For immunofluorescence microscopy the cells were placed on coverslips in medium containing thiabendazole. Prior to fixation the medium was exchanged with Soerensen phosphate buffer for 10 to 30 seconds to induce the formation of microtubules.

In vivo electroporation of cells

HS2205 cells were pelleted and washed twice in Soerensen phosphate buffer (1,000 rpm, 5 minutes, 4°C). The cell suspension, the electroporation cuvette (Eurogentec) and all other reagents were kept on ice. The affinity-purified NAB350 or mouse IgGs, diluted in PBS, were added to 10^7 cells to give a final volume of 0.5 ml and pulsed at 300 V, 250 mF. Cells were washed three times in Soerensen phosphate buffer containing 1% sucrose (1,000 rpm, 5 minutes, 4°C). The pellet was suspended in 0.5 ml HS2205 growth medium and the cells were allowed to settle on poly-L-lysine covered coverslips which were placed in 3 ml Petri dishes. After 20 minutes the dishes were filled with medium; 2 to 23 hours after the electroporation the cells were fixed and processed for immunofluorescence microscopy using just the secondary antibody.

In vitro polymerization experiments

The preparation of nucleus/NAB complexes was performed with modifications based on the method of Kuriyama et al. (1982). Briefly, 5 ml of logarithmically growing HS2205 cells were pelleted (1,500

rpm, 5 minutes, 4°C). All subsequent steps were performed at 4°C. The cells were washed three times in Soerensen phosphate buffer in Eppendorf tubes then 50 ml of the cell pellet was transferred to 0.5 ml of lysis buffer II (100 mM Pipes, pH 6.9, 0.25% Triton X-100, 2 mM $MgCl_2$, 3 mM PMSF, 1 mg/ml leupeptin) in an Eppendorf tube. Cells were lysed by vortexing for 2 minutes and the nucleus/NAB complexes were pelleted (Eppendorf tabletop centrifuge, 6,000 rpm, 10 minutes). Porcine brain tubulin was prepared as described elsewhere (Steinberg and Schliwa, 1995). The polymerization experiments were performed using procedures based on those of Mitchison and Kirschner (1984). Briefly, the freshly isolated complexes and tubulin were suspended in 20 ml of 4°C polymerization buffer (0.1 M Pipes, pH 6.9, 0.5 mM $MgSO_4$, 1 mM EGTA, 1 mM GTP). The suspension was warmed to 37°C and the reaction stopped after 5 minutes by the addition of 10 ml 3% glutaraldehyde in polymerization buffer without GTP. The complexes were allowed to sediment on a poly-L-lysine covered coverslip and prepared for immunofluorescence microscopy using formaldehyde fixation: 3.7% formaldehyde in PEM (PHEM without Hepes) buffer, for 30 minutes.

RESULTS

The monoclonal antibody NAB350 identifies the *D. discoideum* centrosome (NAB)

Using a modified standard immunization protocol with nucleus/NAB complexes as immunogen, a stable NAB antibody-producing hybridoma cell line was isolated. The secreted IgG-antibody, called NAB350, exhibited an unusually intense staining of the NAB in immunofluorescence microscopy. The staining was restricted to one dot-like structure in interphase AX2 cells (Fig. 1A). Comparison of this staining pattern with the distribution of microtubules in interphase cells revealed that the point of origin of the microtubule network, the NAB, was stained with NAB350 (Fig. 1B). The NAB-antigen was present during the whole cell cycle but exhibited some changes in its cellular localization during cell cycle progression. During mitosis the immunofluorescence staining was far less intense than in interphase cells. Fig. 2 shows the distribution of the NAB-antigen during mitosis in comparison with the distribution of microtubules and nuclei. During prometaphase the antigen was located near the spindle poles. From metaphase to anaphase (Fig. 2A) the antigen exhibited an additional localization along the microtubules of the mitotic spindle and was no longer concentrated in one dot-like structure. Instead it was also dispersed in the cytoplasm directly adjacent to the NAB. This distribution of the antigen prevailed throughout telophase (Fig. 2D). During cytokinesis the protein was identified only at the spindle poles and no longer in association with the microtubules of the mitotic spindle (Fig. 2G).

NAB350 identifies a protein of 350 kDa and cross reacts with the myosin heavy chain

Western blot analysis of homogenized whole cells and isolated nucleus/NAB complexes of AX2 cells identified a high molecular mass component estimated at 350 kDa. Furthermore, the antibody showed a reaction with a fairly abundant protein of approximately 240 kDa and with some smaller, less abundant polypeptides (Fig. 3A, lanes a1, a3). Comparison of this reaction pattern with that identified by the myosin heavy chain (MHC) antibody mAb96 revealed that the abundant 240 kDa protein was MHC (Fig. 3A, lanes b1, b3; Warrick et al., 1986). To confirm this result, MHC-negative HS2205 cells and isolated HS2205 nucleus/NAB complexes were analyzed in western blots. In these blots only NAB350 identified the 350 kDa antigen (Fig. 3A, lanes a2, a4) whereas no proteins were identified with the MHC-specific mAb96 (Fig. 3A, lanes b2, b4). In western blots the 350 kDa protein is located below the dynein heavy chain (not shown) identified by an antibody against the heavy chain of *D. discoideum* cytoplasmic dynein (Koonce and McIntosh, 1990).

To determine the subcellular distribution of the 350 kDa antigen, AX2 cells were lysed and centrifuged at 100,000 ***g*** for 30 minutes to separate soluble and insoluble cellular constituents. Whereas myosin was present in both fractions (Fig. 3B, lanes 1 and 2), the 350 kDa antigen was found exclusively in the pellet (Fig. 3B, lane 1), suggesting that it is structure-bound and/or structure-forming.

The results from the immunoblot analysis were confirmed by immunofluorescence microscopy. NAB350 clearly localized to the centrosome in interphase HS2205 cells (Fig. 4A), whereas mAb96 did not identify any antigen in these cells (not shown). The cross reaction of NAB350 with MHC can be made visible in immunofluorescence preparations if the AX2 cells are prepared and fixed according to the agar-overlay technique (Yumura et al., 1984). Fig. 5 shows the localization of the antigens identified by the antibodies mAb96 and NAB350 in immunofluorescence preparations of AX2 cells prepared by the agar-overlay technique. The MHC-antibody mAb96 is localized only to the trailing edge of the moving amoebae (Fig. 5A), whereas NAB350 reacts with the cortical MHC as well as the NAB-antigen (Fig. 5C).

Bailly et al. (1992) described a comparable cross reaction for the antibody CTR56 which identifies a 350 kDa antigen in preparations of human centrosomes and also reacts with the heavy chain of conventional myosin in cells and western blots

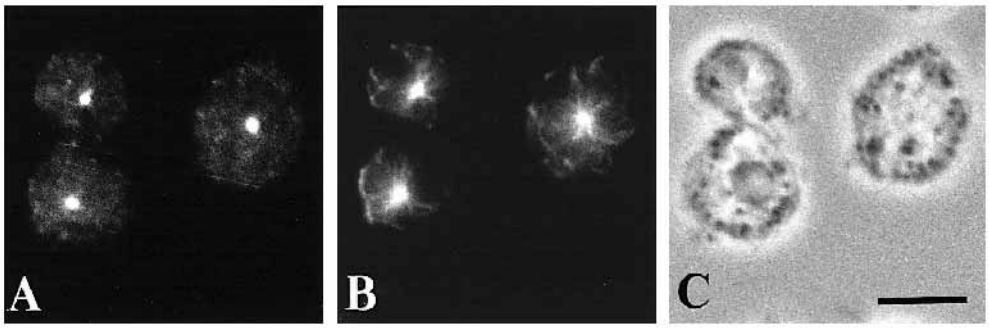

Fig. 1. Immunofluorescence staining of interphase AX2 cells with NAB350. (A) Cells stained with NAB350. (B) Same cells stained with antibody YL 1/2 showing the distribution of microtubules. (C) Corresponding phase contrast picture. The NAB lies in the center of the interphase microtubule network. Bar, 10 μm.

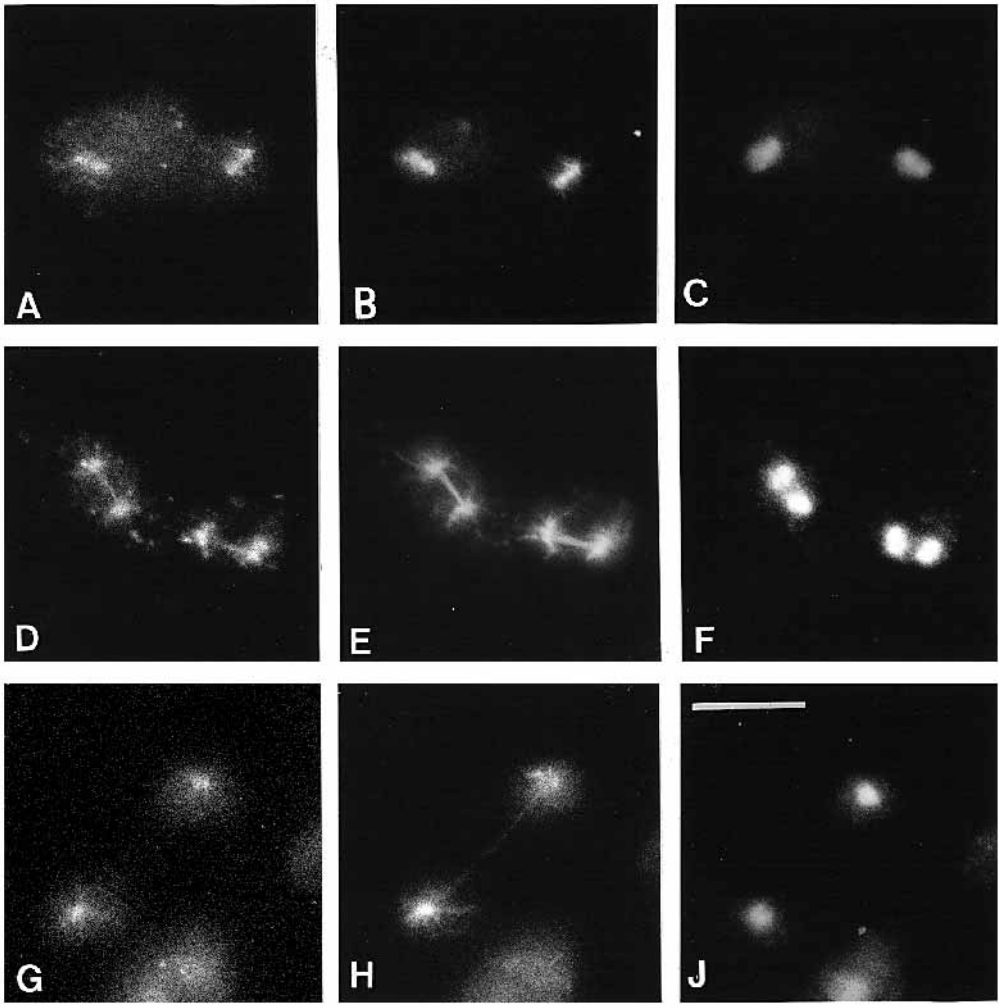

Fig. 2. Immunofluorescence staining of mitotic AX2 cells with NAB350. Cells in (A) anaphase, (D) telophase, and (G) during cytokinesis stained with NAB350. (B,E,H) The corresponding tubulin distribution (staining with YL 1/2). (C,F,J) The corresponding DAPI stained cells revealing the location of DNA. The 350 kDa antigen is localized at the spindle poles during mitosis. Between metaphase and telophase it exhibits an additional localization along the microtubules of the mitotic spindle. Bar, 10 μm.

of centrosomal proteins. However, immunofluorescence investigations did not confirm a possible immunological homology between the 350 kDa CTR56 and NAB350 antigens. NAB350 did not identify a centrosomal antigen in human cells or centrosomes (in collaboration with M. Bornens), nor did CTR56 recognize an NAB-antigen in *D. discoideum* cells.

Localization of the 350 kDa antigen by immunogold electron microscopy and its relative stability towards urea- and KCl-treatment

The localization of the 350 kDa antigen was studied in the electron microscope by immunogold labeling of isolated AX2 nucleus/NAB complexes. Fig. 6 shows an overview of a NAB in an intact AX2 cell for comparison. The localization of the gold particles identified the 350 kDa antigen as part of the NAB corona, a less electron-dense 'fuzzy' layer covering the dense centrosomal core (Bottini and Roos, 1992; Kuriyama et al., 1982; Omura and Fukui, 1985) (Fig. 7A). To study the stability of the antigen's association with the NAB corona, isolated AX2 nucleus/NAB complexes were treated with urea or KCl. Detailed immunofluorescence microscopic analysis demonstrated that the association of the 350 kDa antigen with the NAB corona was relatively resistant towards treatment with these two agents: up to 0.4 M KCl or 4 M urea, the NAB350 fluorescence was unaffected, but it virtually vanished at 1 M KCl and 5 M urea (not shown). At these concentrations most of the 350 kDa antigen is released into the supernatant. Electron microscopy of nuclei treated with 0.6 M KCl or 2 M urea caused a partial or complete solubilization of the box-shaped central core of the NAB, whereas the corona and the associated 350 kDa antigen could still be distinguished (Fig. 7B,C). The 350 kDa antigen therefore shows a fairly stable association with that part of the NAB that is responsible for the nucleation of microtubules.

NAB350 identifies induced, cytoplasmic MTOCs

The incubation of *D. discoideum* cells with thiabendazole results in cells that contain, in addition to the NABs, cytoplasmic MTOCs not associated with the nucleus (Kitanishi et al., 1984; Kitanishi-Yumura et al., 1985). Immediately upon removal of the compound, the cytoplasmic MTOCs start to nucleate microtubules (Kitanishi-Yumura et al., 1985). The location and number of these cytoplasmic MTOCs can therefore be determined by immunofluorescence analysis of these developing microtubule asters. In both AX2 and HS2205 cells the 350 kDa antigen was identified in the centers of all aster-shaped microtubule structures by immunofluorescence microscopy (Fig. 8A,B). The cytoplasmic MTOCs were of variable size, most of them being smaller than the nucleus-associated NABs. Clearly, however, the 350 kDa antigen was

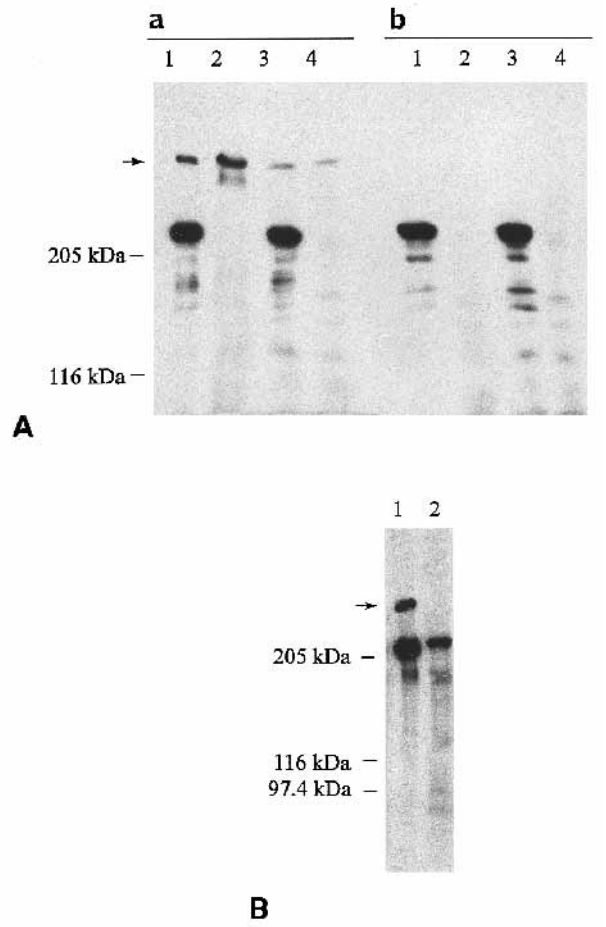

Fig. 3. (A) Western blot analysis of the *D. discoideum* proteins separated in a 4.5% SDS-PAGE. Blot (a) was incubated with NAB350, blot (b) with the MHC-antibody mAb96. The separated proteins in the lanes were: (1) 4×10^6 AX2 nucleus/NAB complexes, (2) 4×10^6 HS2205 nucleus/NAB complexes, (3) 4×10^5 AX2 cells, and (4) 4×10^5 HS2205 cells. NAB350 recognizes a 350 kDa antigen (arrow) and cross reacts with MHC. The size of the molecular mass marker is indicated on the left. (B) Western blot analysis of the subcellular distribution of the 350 kDa antigen. AX2 cells were freeze-thawed in liquid nitrogen and centrifuged at 100,000 ***g*** for 30 minutes. The proteins were separated on a 4.5% SDS-PAG and the western blot was incubated with NAB350. The 350 kDa antigen (arrow) is found exclusively in the pellet (lane 1) and not the supernatant (lane 2).

found to be associated with all structures, both nucleus-associated and cytoplasmic, capable of nucleating microtubules.

NAB350 does not interfere with microtubule nucleation in vitro

To determine whether NAB350 affects microtubule nucleation of NABs in vitro, isolated nucleus/NAB complexes of MHC-negative HS2205 cells were incubated with NAB350 at a concentration of 1.6 mg/ml, followed by purified porcine brain tubulin. In control experiments, the antibody incubation step was ommitted. The number of induced microtubules was counted with the aid of immunofluorescence microscopy after staining the samples with the α-tubulin antibody YL 1/2. The nucleation of microtubules from isolated NABs depended on the concentration of tubulin used, being more efficient at higher tubulin concentrations. However, no difference in the number of nucleated microtubules was detected with or without antibody incubation at tubulin concentrations of 0.75, 1, and 1.125 mg/ml (n=162 to 269) (not shown). The antibody bound to the NAB of these isolated nucleus/NAB complexes, as demonstrated in Fig. 9. It is unlikely that microtubule formation in these experiments is due to the elongation of pre-existing short microtubule segments; neither immunofluorescence nor electron microscopy demonstrated the presence of tubulin or microtubule 'stubs' at the NAB. We suggest, therefore, that NAB350 does not interfere with microtubule nucleation from *D. discoideum* NABs in vitro.

In vivo study of the function of the 350 kDa antigen

To gain some insight into the function of the 350 kDa antigen, an in vivo antibody-blocking experiment was devised. Since microinjection of single amoebae is rather tedious and can be performed on a limited number of cells only, we adapted an electroporation protocol, normally used for the transfection of cells, in order to introduce NAB350 into HS2205 cells. The cells were fixed for immunofluorescence microscopy at various times between 2 and 23 hours after electroporation. The uptake of antibody by endocytosis was not observed in control experiments in which the cells were exposed to NAB350-containing buffer without electroporation.

The proportion of cells with immunologically localized NABs was dependent on the concentration of antibody in the surrounding buffer during the electroporation experiment. Up

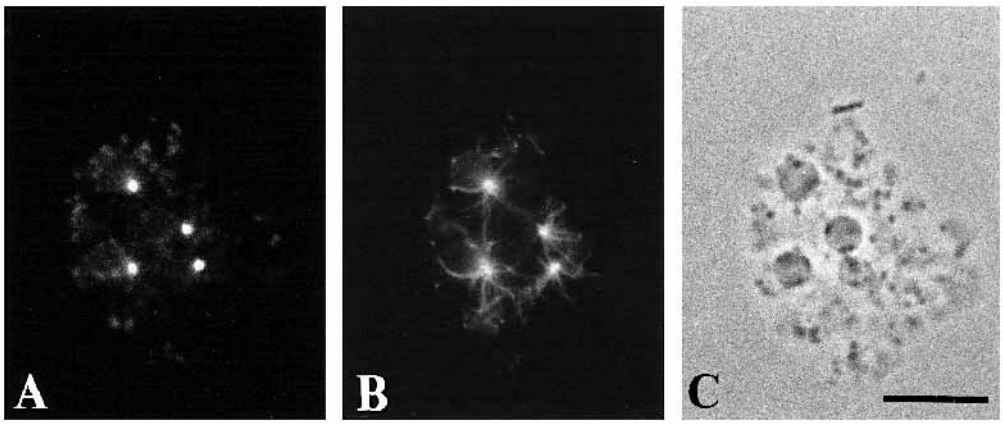

Fig. 4. Localization of the 350 kDa antigen at the NAB of a HS2205 cell. (A) Cell stained with NAB350. (B) Same cell stained with YL 1/2 showing the tubulin distribution. (C) Corresponding phase contrast picture. Bar, 10 μm.

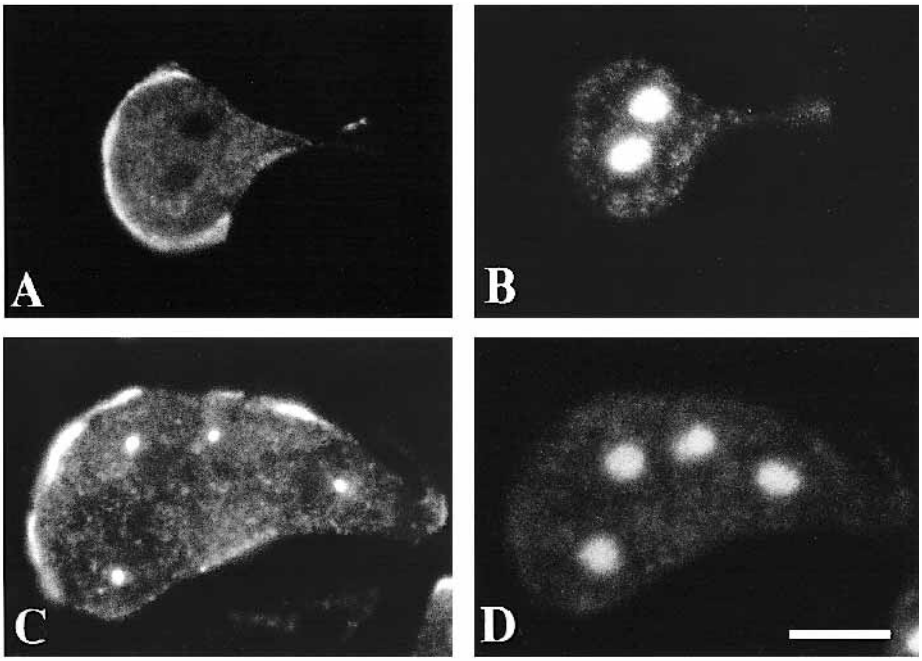

Fig. 5. Localization of the NAB350 antigens in AX2 cells prepared by the agar-overlay technique. (A) Cell stained with mAb96. (C) Cell stained with NAB350. (B and D) The corresponding DAPI staining. The cross reaction between NAB350 and MHC can be made visible by the agar-overlay technique. Only NAB350 recognizes the NAB antigen. Bar, 10 μm.

to 12.6% of the cells were labeled if 0.5 mg/ml NAB350 was used (2 experiments, cells examined at at least 3 time intervals between 2 and 22 hours after electroporation; at least 200 cells were examined for each data point). The percentage of labeled cells at antibody concentrations of 0.02 mg/ml or 0.1 mg/ml was between 2 and 6% (3 experiments each, examined at various time intervals between 2 and 23 hours, at least 200 cells were examined for each data point). As a negative control cells were electroporated with identical concentrations of mouse IgGs.

Whereas the majority of cells electroporated with NAB350 did not show any change in the distribution of microtubules, the number of NABs or the size of their nuclei, an average of 1 in 2,074 cells (at 0.1 mg/ml antibody concentration) or 1 in 1,753 cells (at 0.5 mg/ml NAB350 antibody) with an immunologically localized NAB did not contain microtubules (Fig. 10; 3 independent experiments). These numbers are consistent with the idea that a deleterious effect caused by the antibody is only observed if NAB350 is introduced during cell division. Since mitotic cells constitute about 1% of an exponentially growing culture, and since electroporation is successful in roughly 5 to 10% of these mitotic cells (see previous paragraph), one would expect between 1 in 1,000 and 1 in 2,000 cells to be affected. In control cells, which were electroporated with mouse IgGs, no cells without microtubules were observed.

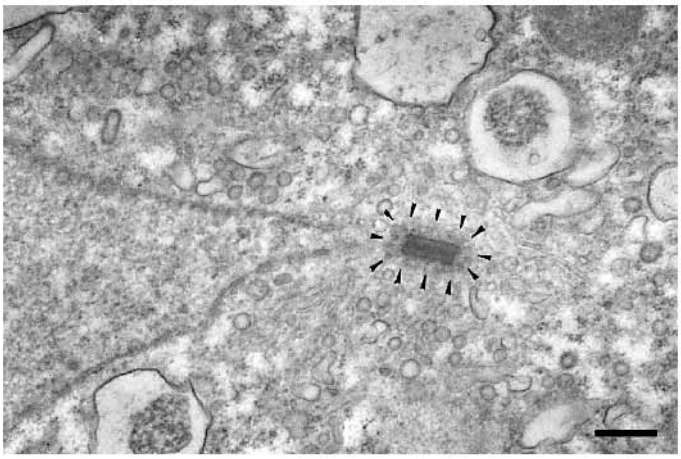

Fig. 6. Overwiew of a NAB in an intact *D. discoideum* amoeba, showing the dark centrosomal core and the corona (delineated by small arrowheads). The NAB is connected to the tapered end of the nucleus. Bar, 0.5 μm.

DISCUSSION

Using a monoclonal antibody approach, we have identified a high molecular mass component of the *D. discoideum* centrosome (NAB). This protein, with a molecular mass of 350 kDa, localizes to the peripheral amorphous layer of the centrosome, the corona, from which microtubules emerge. Functional studies in vitro and in vivo suggest that this protein is unlikely to be directly involved in microtubule nucleation but may be required for the maintenance of the structural fidelity of the NAB. Though the antibody we have generated also crossreacts with the *D. discoideum* myosin heavy chain, we have demonstrated, using a myosin null mutant, that the 350 kDa antigen is localized primarily, if not exclusively, to the NAB. Thus it appears to be the only centrosome-specific component aside from gamma-tubulin (U. Euteneuer, unpublished observations) that has been identified in *D. discoideum*. Previously, other antibodies such as CHO3 (Sellitto and Kuriyama, 1988) or a series of 6 antibodies raised against nucleus/NAB complexes (Sellitto et al., 1992) were shown to label the *D. discoideum* NAB, but they react with multiple polypeptide bands in western blots of whole cells and/or stain nuclei, microtubules or granules in addition to centrosomes in immunofluorescence microscopy.

The behavior of the 350 kDa antigen as revealed by

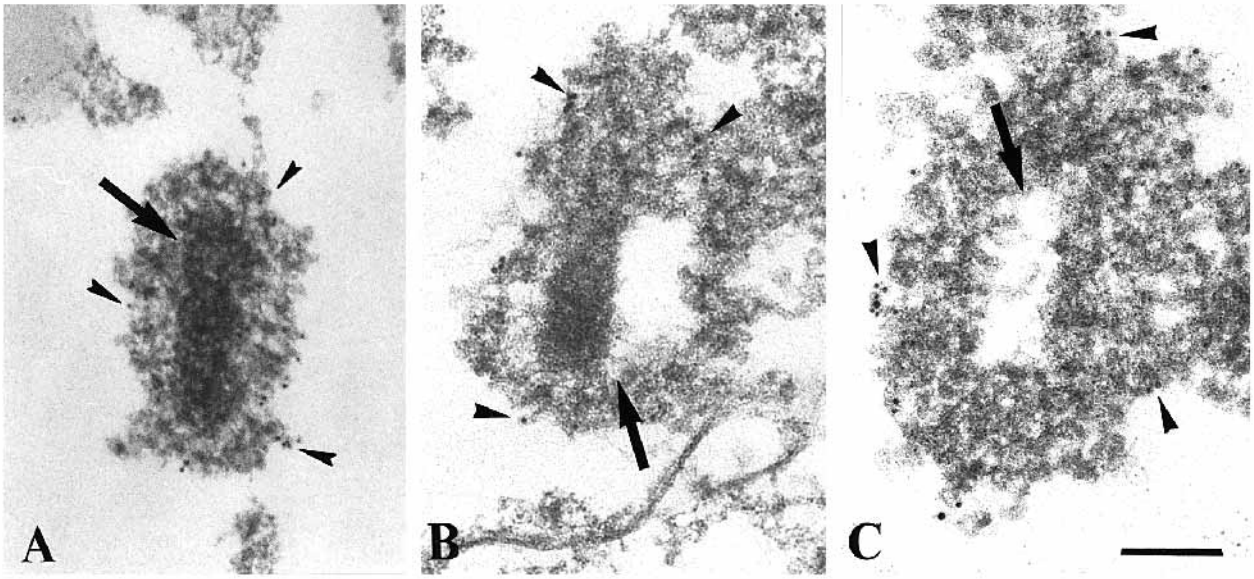

Fig. 7. Electron microscopic determination of the localization of the 350 kDa antigen on nucleus/NAB complexes. Immunogold localization of the 350 kDa antigen on (A) an isolated nucleus/NAB complex, (B) a complex treated with 0.6 M KCl, and (C) a complex treated with 2 M urea. The 350 kDa antigen is part of the NAB corona. Arrowheads, localization of gold particles; arrows, localization of NAB core. Bar, 0.2 μm.

immunofluorescence microscopy bears some resemblance to that of two centrosomal components of mammalian cells, the CTR56-antigen (Bailly et al., 1992) and an antigen recognized by rabbit serum 0013 (Gosti-Testu et al., 1986; Keryer et al., 1993; Komesli et al., 1989). Both identify centrosomal components of about 350 kDa. Moreover, the CTR56 monoclonal antibody also crossreacts with the MHC in human cells (Bailly et al., 1992). CTR56 does not recognize a *D. discoideum* polypeptide, however. Likewise, the serum 0013 antigen which binds to the regulatory subunit of the cAMP-dependent protein kinase II in human cells (Keryer et al., 1993), seems to be restricted to mammalian cell lines (Komesli et al., 1989; M. Bornens, unpublished observations). Whether the CTR56 and serum 0013 antigens represent the same protein has not been determined yet. Based on the identical reactivity of CTR56 and NAB350 with their respective 350 kDa antigens as well as MHCs, it is conceivable that the *D. discoideum* and human 350 kDa antigens represent functionally equivalent proteins of these two differently structured types of centrosomes.

The crossreaction between NAB350 and the *D. discoideum* MHC may well be based on a structural homology. A differential immunological screening of a *D. discoideum* AX3 λgt

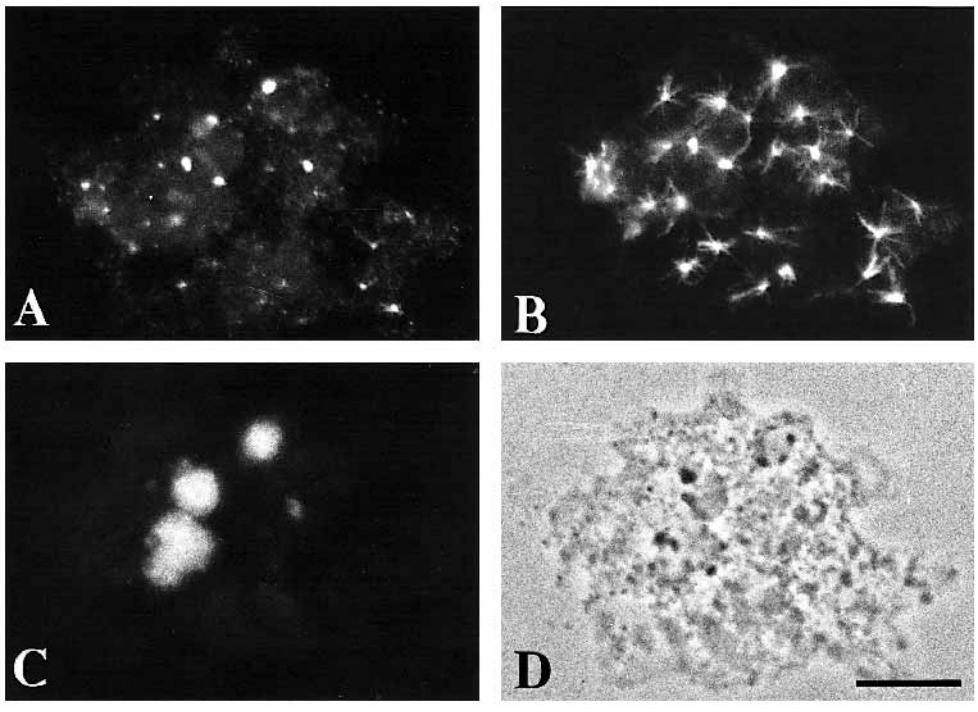

Fig. 8. The 350 kDa antigen is part of thiabendazole-induced, cytoplasmic MTOCs in AX2 cells. (A) Cells stained with NAB350. (B) Same cell stained with YL 1/2 showing the tubulin distribution. (C and D) Corresponding DAPI stain and phase contrast picture. Bar, 10 μm.

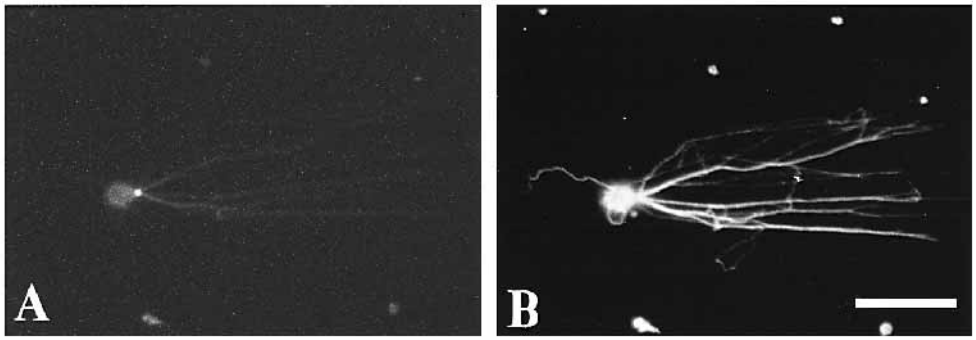

Fig. 9. Microtubule polymerization from an HS2205 nucleus/NAB complex after incubation with NAB350. (A) The NAB is labeled with NAB350. (B) Staining with YL 1/2 shows the polymerized microtubules which originate from the NAB. Bar, 10 μm.

11 cDNA library with two MHC antibodies (Claviez et al., 1982; Pagh and Gerisch, 1986) and the NAB350 antibody was performed (data not shown). These studies, which did not yet identify the gene for the 350 kDa antigen, showed that the epitope that is responsible for the crossreaction most probably is localized to a 50 amino acid stretch (between positions 1,622 and 1,671) of the coiled-coil tail of *D. discoideum* MHC. We speculate that the NAB350 antibody recognizes a coiled-coil segment in the 350 kDa antigen as well.

Though western blot analysis demonstrates the presence of MHC at isolated nulceus/NAB complexes, it is unlikely that myosin is an intrinsic, essential component of the NAB since *D. discoideum* MHC-null mutants are able to perform all centrosome-associated tasks. Furthermore, MHC antibodies do not decorate the NAB in wild-type AX2 cells. As in mammalian centrosome preparations, myosin may associate with nucleus/NAB complexes during the isolation process, probably due to non-specific intractions (Klotz et al., 1990; Komesli et al., 1989; Bailly et al., 1992). It is also likely that the fraction of nucleus/NAB complexes is still contaminated with fragments of the myosin-rich cell cortex, which cannot be separated from the nuclei in these fractions.

The immunoelectron-microscopic studies identified the 350 kDa antigen as a component of the NAB corona that covers the electron-dense box-shaped core. Hydrophobic interactions may in part be responsible for the maintenance of the structural integrity of the corona. For example, the 350 kDa antigen can be dissociated from the NAB with 6 M urea (not shown). This behavior is reminiscent of two *S. cerevisiae* spindle pole body components, Nuf2 and Nuf1/Spc110, which can only be solubilized by 8 M urea (Osborne et al., 1994). Whereas the corona, including the 350 kDa antigen, is relatively stable towards extraction with 2 M urea and 0.6 M KCl, the core structure is patially or completely dissolved upon these treatments. Analogous observations were made with human centrosomes where 2 M urea partially removes or destroys the centrioles, while the pericentriolar matrix surrounding them resists this treatment (Klotz et al., 1990). It may be too early to speculate that the corona and the pericentriolar matrix are homologous structures, but their similar behavior during extraction and their apparent involvement in the nucleation of microtubules certainly is intriguing.

The localization of the 350 kDa antigen in the microtubule-organizing corona of the NAB (Omura and Fukui, 1985) suggests a role in the nucleation, anchorage, or regulation of microtubule asembly, or an involvement in the structural organization of the corona. These two possibilities are supported by the antigen's association with cytoplasmic MTOCs in thiabendazole-treated cells. In vitro assays using isolated HS2205 nucleus/NAB complexes incubated with NAB350 antibody did not reveal any detrimental effect on microtubule nucleation and polymerization. This does not, however, mean that the 350 kDa antigen might not be involved in these processes as the antibody may bind at a site that does not interfere with the molecule's function. On the other hand, electroporation of living cells with NAB350 appeared to interfere with microtubule nucleation in some cells. The relative rareness of the observed effect suggests that interphase NABs are not influenced by antibody electroporation, in accordance with the results of the in vitro nucleation assay. The number of cells affected is, however, consistent with the idea that the electroporated antibody somehow interferes with NAB associated activities in mitosis, a phase of the cell cycle where NAB350 reveals a structural reorganization of the 350 kDa antigen. Experiments on synchronized *D. discoideum* amoebae may help support this hypothesis, but so far our attempts at cell sychronization resulted in only a minor increase in mitotic cells. In any case, both the in vitro and in vivo observations should be interpreted with some caution since it is not known which of the functions of the 350 kDa antigen our antibody might perturb.

In summary, we have identified a novel component of the NAB that most likely is involved in the maintenance of the structural and possibly also the functional integrity of the NAB

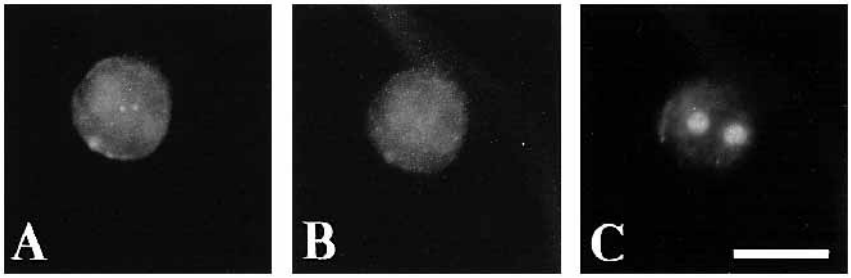

Fig. 10. Microtubule-free HS2205 cell after electroporation with NAB350. (A) Cell stained with NAB350. (B) Staining with YL 1/2 shows the absence of microtubules. (C) DAPI stain of the nuclei. Bar, 10 μm.

corona. Further studies are aimed at the molecular characterization of this minor, yet important component of the *D. discoideum* centrosome and the identification of the molecules it interacts with.

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