

The Golgi Apparatus Segregates from the Lysosomal/Acrosomal Vesicle during Rhesus Spermogenesis: Structural Alterations

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The acrosome is an acidic secretory vesicle containing hydrolytic enzymes that are involved in the sperm's passage across the zona pellucida. Imaging of the acrosomal vesicle and the Golgi apparatus in live rhesus monkey spermatids was accomplished by using the vital fluorescent probe LysoTracker DND-26. Concurrently, the dynamics of living spermatid mitochondria was visualized using the specific probe MitoTracker CMTRos and LysoTracker DND-26 detected the acrosomal vesicle from its formation through spermatid differentiation. LysoTracker DND-26 also labeled the Golgi apparatus in spermatogenic cells. In spermatocytes the Golgi is spherical and, in round spermatids, it is localized over the acrosomal vesicle, as confirmed by using polyclonal antibodies against Golgin-95/GM130, Golgin-97, and Golgin-160. Using both live LysoTracker DND-26 imaging and Golgi antibodies, we found that the Golgi apparatus is cast off from the acrosomal vesicle and migrates toward the sperm tail in elongated spermatids. The Golgi is discarded in the cytoplasmic droplet and is undetectable in mature ejaculated spermatozoa. The combined utilization of three vital fluorescent probes (Hoechst 33342, LysoTracker DND-26, and MitoTracker CMTRos) permits the dynamic imaging of four organelles during primate spermogenesis: the nucleus, the mitochondria, the acrosomal vesicle, and the Golgi apparatus. © 2000 Academic Press

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INTRODUCTION

Acrosome biogenesis is essential for fertilization and for the initiation of development (for reviews see de Kretser and Kerr, 1988; Barros *et al.*, 1996; Tulsiani *et al.*, 1998). The synthesis of the acrosomal enzyme acrosin, and probably many other components of the acrosome, starts at the pachytene stage (Kashiwabara *et al.*, 1990; Escalier *et al.*, 1991). Ultrastructural studies report that acrosin is sorted and packed into an electron-dense granule within the proacrosomal vesicles (Escalier *et al.*, 1991). In spermatids,

the proacrosomal vesicles fuse to each other and attach to the assembled perinuclear theca, forming the acrosomal vesicle (Susi *et al.*, 1971; Sinowatz and Wrobel, 1981). The acrosomal vesicle contains an electron-dense acrosomal granule that gives rise to the acrosomal matrix of mature spermatozoa (Mansouri *et al.*, 1983; Flörke *et al.*, 1983; Westbrook-Case *et al.*, 1995). At this stage, the Golgi apparatus is located over the acrosomal vesicle, and many vesicles bud from it and fuse with the acrosomal vesicle membrane (Susi *et al.*, 1971; Hermo *et al.*, 1980; Sinowatz and Wrobel, 1981). During these stages of spermatid differentiation (cap and acrosome phase), the acrosomal vesicle spreads radially over one-third and, eventually, one-half of the lengthening and compacting nucleus (Burgos and Fawcett, 1955; Fawcett, 1975; Holstein, 1976). After completing the production of acrosomal proteins, the Golgi apparatus separates from the acrosomal vesicle and starts to migrate

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toward the caudal portion of elongating spermatids (Bowen, 1922; Susi *et al.*, 1971). Finally, the Golgi stacks fragment is possibly digested by autophagocytosis in the discarded cytoplasmic droplet (Dietert, 1966; Susi *et al.*, 1971).

The Golgi apparatus in young rat spermatids consists of a compact hemispherical mass next to the developing acrosomal vesicle, with three to nine parallel saccules perforated with pores of various dimensions (Herms *et al.*, 1980). Whereas the cortex (or cis-face) of this hemisphere is made up of stacks of saccules, the mature (or trans-face) side contains only a few membranous elements with a wider lumen (Susi *et al.*, 1971; Herms *et al.*, 1980). The area in between the trans-face and the acrosome (termed the medulla) contains numerous vesicular and tubular profiles of various sizes (Mollenhauer *et al.*, 1976; Burgos and Gutierrez, 1986). β -COP and clathrin-coated vesicles have been identified at the trans-Golgi reticulum in round spermatids, though their role in either anterograde or retrograde transport has not been elucidated (Burgos and Gutierrez, 1986; Martinez-Menarguez *et al.*, 1996b). Syntaxin and vesicle-associated membrane protein (VAMP) are components of the SNARE [soluble *N*-ethylmaleimide-sensitive (NSF) receptor] hypothesis fusion machinery that are involved during vesicle targeting, docking, and fusion between the donor and acceptor membrane (Ungerman *et al.*, 1998). These proteins have been identified in many cell types, and it is believed that they function in intra-Golgi as well as Golgi-plasma membrane vesicle fusion (Nichols and Pelham, 1998). SNARE proteins are present in mature sea urchin sperm and oocytes and probably have a role during the exocytotic process of both the cortical granules in the egg and the acrosome reaction in the sperm (Conner *et al.*, 1997; Schulz *et al.*, 1997, 1998). The characterization, localization, and role of this fusion machinery during spermatogenesis have not yet been determined.

After the acrosome reaction (an exocytotic event resulting from fusion between the outer acrosomal membrane and the plasma membrane), there is an increase in the intracellular pH (pHi) thought to activate a pH-dependent Ca^{2+} pump (Santi *et al.*, 1998). This rise in pHi will in turn activate the fusion machinery between both membranes. Since the pH-sensitive fluorescent probes used in these studies stain the entire sperm head, we cannot discriminate the contribution of the intraacrosomal pH (pHa) and the cytoplasmic pH to this process. It is believed that the function of the low pHa is to prevent a precocious activation of the proenzymes, such as proacrosin, that will trigger both the acrosomal matrix and the zona pellucida degradation (Moreno *et al.*, 1998). However, this low pHa might also play a role during spermatogenesis by inducing the aggregation and packing of acrosomal proteins, similar to that postulated for the formation of neuroendocrine secretory granules (reviewed by Tooze, 1998). A first approach to determine such a role may be to determine the pHa of the acrosomal vesicle in round spermatids.

Human autoantibodies have been used to characterize several Golgi complex proteins, including Giantin (macro-

Golgin; Seelig *et al.*, 1994; Linstedt and Hauri, 1993), Golgin-245 (Fritzler *et al.*, 1995), Golgin-95 and -160 (Fritzler *et al.*, 1995), and Golgin-97 (Griffith *et al.*, 1997). The family of Golgin antigens shares a number of interesting characteristics that make them a valuable tool in studying the Golgi apparatus. First, the antigens localize to the Golgi apparatus in a brefeldin A-dependent manner. Second, they contain several coiled-coil domains, which are shared by other proteins involved in intracellular trafficking (reviewed by Nichols and Pelham, 1998). Third, Golgin-97 and -245 contain a domain homologous to the peptide hormones and neuropeptides known as granins, suggesting a role in the processing or control of vesicular content (Huttner *et al.*, 1991). Fourth, mammalian Golgi proteins Golgin-97, Golgin-245/p230 have a conserved domain of about 50 amino acids at their carboxyl termini that target these proteins to the Golgi apparatus (Kjer-Nielsen *et al.*, 1999; Munro and Nichols, 1999). This GRIP domain contains a conserved tyrosine residue that is involved in the binding of these proteins to rab6, one of the small GTP-binding proteins involved in intra-Golgi vesicular traffic (Barr, 1999). It is possible that this family of coiled-coil proteins functions in Rab6-regulated membrane-tethering events.

This study visualizes acrosome biogenesis in living rhesus monkey spermatids. Since this organelle is slightly acidic (Cross and Razy-Faulkner, 1997) we utilized the permanent vital fluorescent acidotropic probes LysoTrackers (Haller *et al.*, 1996). They consist of a fluorophore moiety linked to a weak base that is only partially protonated at neutral pH and freely permeable to cell membranes (Molecular Probes, Eugene, OR). LysoTracker DND-26 has previously been used to assess the acrosomal integrity of bovine spermatozoa after cryopreservation (Thomas *et al.*, 1997). We show that LysoTracker DND-26 detects the acrosomal vesicle in round spermatids, as well as the acrosome in mature spermatozoa. LysoTracker DND-26 also labeled the Golgi apparatus of round and elongated rhesus monkey spermatids, confirmed by colocalization with anti-Golgin-97, Golgin-95/GM130, and Golgin-160 antibodies. This probe may therefore be used to study Golgi apparatus dynamics in living spermatids.

MATERIALS AND METHODS

Unless otherwise stated, all chemicals were purchased from Sigma Chemical Company (St. Louis, MO). Antibodies against Golgin-97, -94, and -160 were prepared as described previously (Fritzler *et al.*, 1995; Griffith *et al.*, 1997), as were antibodies against syntaxin and VAMP (Conner *et al.*, 1997; Schulz *et al.*, 1997). Dr. Claudio Barros (Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile, Santiago, Chile) generously donated monoclonal antibodies against acrosin.

Isolation of Spermatogenic Cells and Vital Labeling

Rhesus monkey testes were obtained from males undergoing necropsy for reasons unrelated to fertility. Testicular cells were dissected and transferred to a petri dish filled with TALP-Hepes (Bavister *et al.*, 1983; Boatman, 1987; modified Tyrode-lactate medium with pyruvate and albumin: 114 mM NaCl, 3.2 mM KCl, 2 mM CaCl₂, 0.5 mM MgCl₂, 25 mM NaHCO₃, 0.4 mM NaH₂PO₄, 10 mM sodium lactate, 6.5 IU penicillin, 25 µg/ml gentamicin, 3 mg/ml fatty-acid-free bovine serum albumin, 0.2 mM pyruvate, buffered with 10 mM Hepes at pH 7.4) and minced with two fine forceps. The minced tissue was filtered through a fine mesh to remove the tissue debris, and the cell suspension was centrifuged for 5 min at 700g. The pellet was resuspended in 12 ml of warm TALP-Hepes and centrifuged again for 5 min at 700g. Twenty-five microliters of the loose final pellet was then resuspended in 975 ml of TALP-Hepes for MitoTracker and LysoTracker DND-26 labeling. The primary stock of 1 mM MitoTracker CMTMRos (Molecular Probes, Eugene, OR) was prepared in dimethyl sulfoxide (DMSO). A secondary stock of 100 mM MitoTracker was made from the primary stock in 37°C KMT medium (100 mM KCl, 2 mM MgCl₂, 10 mM Tris-HCl, pH 7.0). The stock was added to a 1-ml suspension of testicular cells to obtain a final concentration of 400 nM. In some cases, the cells were incubated for 1 h with a vital lysosome specific probe LysoTracker green DND-26 (Molecular Probes) at the concentration of 1 mM combined with 200 nM MitoTracker CMTMRos and 5 mg/ml Hoechst 33342 at 37°C. After incubation, the cells were collected by a 5-min centrifugation at 700g and washed by resuspension and centrifugation in 12 ml of TALP-Hepes medium (Sutovsky *et al.*, 1999). Since the LysoTracker green DND-26 label is lost after fixation, the cells were mounted onto coverslips and imaging immediately after labeling.

Immunofluorescence Microscopy

Sperm or spermatogenic cells were attached to poly-L-lysine-coated microscopy coverslips in 1 ml of KMT medium and fixed for 1 h with 2% formaldehyde in 0.1 M phosphate-buffered saline (PBS; pH 7.2). The coverslips were rinsed in PBS and permeabilized for 1 h in 1% Triton X-100 in PBS. Nonspecific antibody cross-reactions were blocked by a 1-h preincubation in 0.1 M PBS containing 2% BSA and 130 mM glycine. The coverslips were incubated with the antibodies against the Golgi apparatus (Golgin-97, Golgin-95/GM130, and Golgin-160) in a dilution of 1:200 (Fritzler *et al.*, 1995) and anti-acrosin (a mixture of four different monoclonal antibodies) in a dilution of 1:200 (Valdivia *et al.*, 1994) for 2 h at room temperature and then diluted in PBS-2% BSA. After three rinses, samples were incubated for 40 min with either TRITC- or FITC-conjugated appropriate secondary antibodies diluted in PBS containing 0.05% NP-40. Five micrograms per milliliter of 4,6-diamidino-2-phenylindole (DAPI; Molecular Probes) was added 10 min before the end of incubation. The coverslips were rinsed three times and mounted in a drop of VectaShield mounting medium (Vector Labs, Burlingame, CA).

Coverslips were examined using a Zeiss Axiophot epifluorescence microscope and photographed using a chilled CCD camera (Princeton Instruments Inc., Trenton, NJ) operated by Metamorph software. Original data were archived on recordable CDs. Images were pseudocolored and the contrast was enhanced using Adobe Photoshop 4.0 software (Adobe Systems Inc., Mountain View, CA) and printed on a Sony UP-D 8800 color video printer (Sony Corporation, Park Ridge, NJ).

Cell Extract Preparation

Sperm or testicular cells were washed twice by centrifugation in 0.1 M PBS. The pellet was resuspended in 0.5 ml of a buffer containing 1 M NaCl, 20 mM imidazole (pH 6.0), 1% Triton X-100, 1 mM ethylenediaminetetraacetic acid (EDTA), 5 mM benzamidine HCl, 5 mg/ml leupeptin, and 1 mg/ml pepstatin A (Noland *et al.*, 1994). This solution was incubated overnight at 4°C and then centrifuged for 10 min at 12,000g in a microcentrifuge (IEC, Micromax RF; International Equipment Co., Needham Heights, MA). The supernatant containing the solubilized proteins was stored at -20°C.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blot

The presence of acrosin and Golgi apparatus protein antigens in sperm and testicular extracts was determined by one-dimensional SDS-PAGE (Laemmli, 1970). Protein was determined by using the bicinchoninic acid method (Pierce Chemical Company, Rockford, IL). Samples for analysis were run on a 10% SDS-PAGE under reducing and denaturing conditions and then transferred to Hybond sheets using a dry system at 0.8 mA/cm². Hybond sheets were blocked with 2% PBS-BSA for 1 h and incubated overnight at 4°C with either the antibodies against Golgi apparatus (Golgin-97, Golgin-95/GM130, and Golgin-160) at a dilution of 1:1000 or a mixture of human acrosin monoclonal antibodies C5F10, A8C10, and C2B10 (Moreno *et al.*, 1998). After extensive washing, they were incubated with either anti-mouse or anti-rabbit goat IgG tagged with horseradish peroxidase. The bands were developed using the ECL Plus system (Amersham, Arlington Heights, IL).

Acrosin Activity

Acrosin activity was measured spectrophotometrically at 25°C by following the hydrolysis of *N*-benzoyl-L-arginine ethyl ester (BAEE) and by the addition of 50 or 100 ml of the enzyme solution at 253 nm. The assays were performed in 3-ml volumes, utilizing a substrate mixture of 50 mM Tris, 50 mM CaCl₂, and 500 mM BAEE at pH 8.0. A molar absorption difference of 1150 M⁻¹ cm⁻¹ was used to convert changes in optical density to micromoles of BAEE hydrolyzed (Whittaker and Bendre, 1965). One international unit (IU) of activity was defined as that amount of acrosin hydrolyzing 1 µmol BAEE/min at 25°C.

RESULTS

LysoTracker DN-26 Labeling and Acrosin Characterization in Rhesus Sperm

Freshly ejaculated rhesus sperm prelabeled with 1 mM LysoTracker DN-26 displayed intense and uniform labeling of the acrosome (Fig. 1A). This label disappeared after incubation with 10 µM the calcium ionophore ionomycin (Fig. 1A, inset). Ionomycin disrupts the pH gradient across membranes and induces vesicle exocytosis in different cell types (Fissore *et al.*, 1996; Peters and Mayer, 1998). To confirm LysoTracker DND-26 labeling of the acrosomal vesicle, a mixture of monoclonal antibodies against human acrosin was used (Valdivia *et al.*, 1994). Staining with these

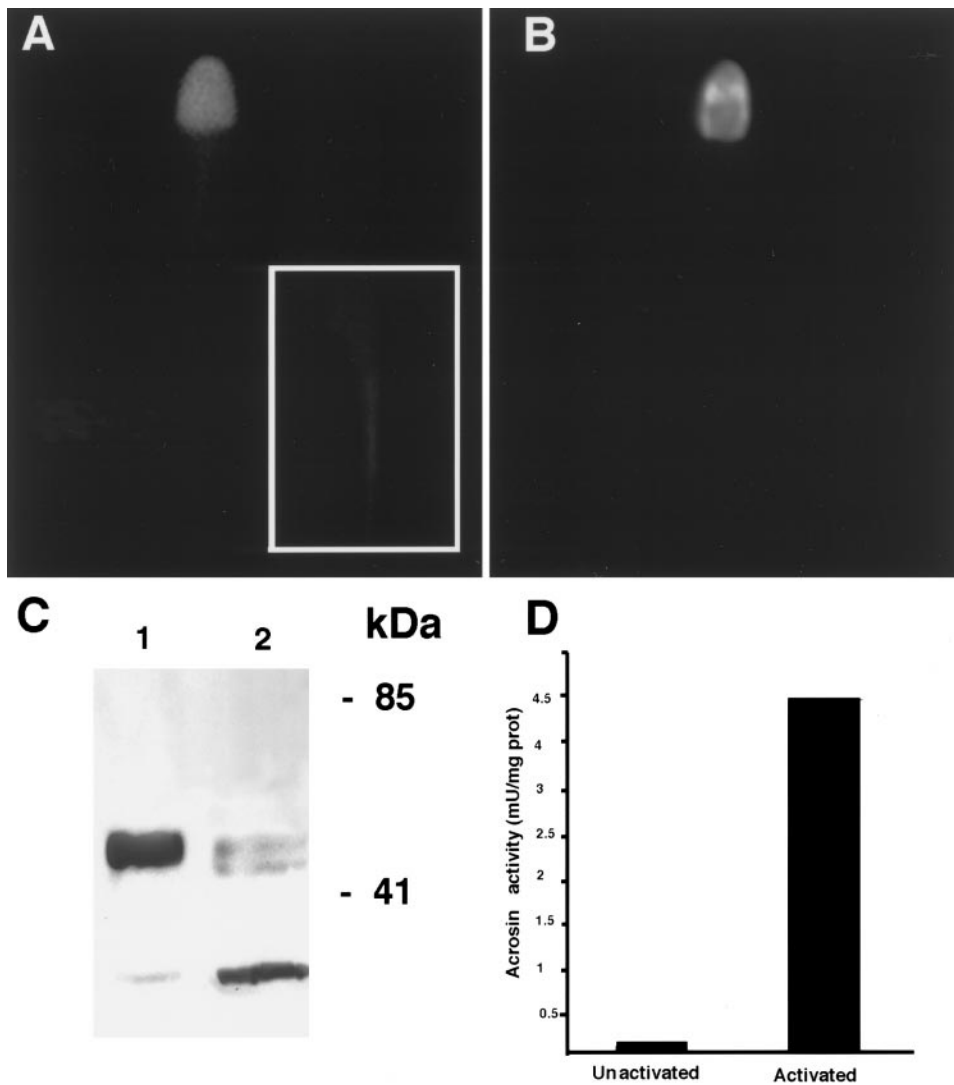
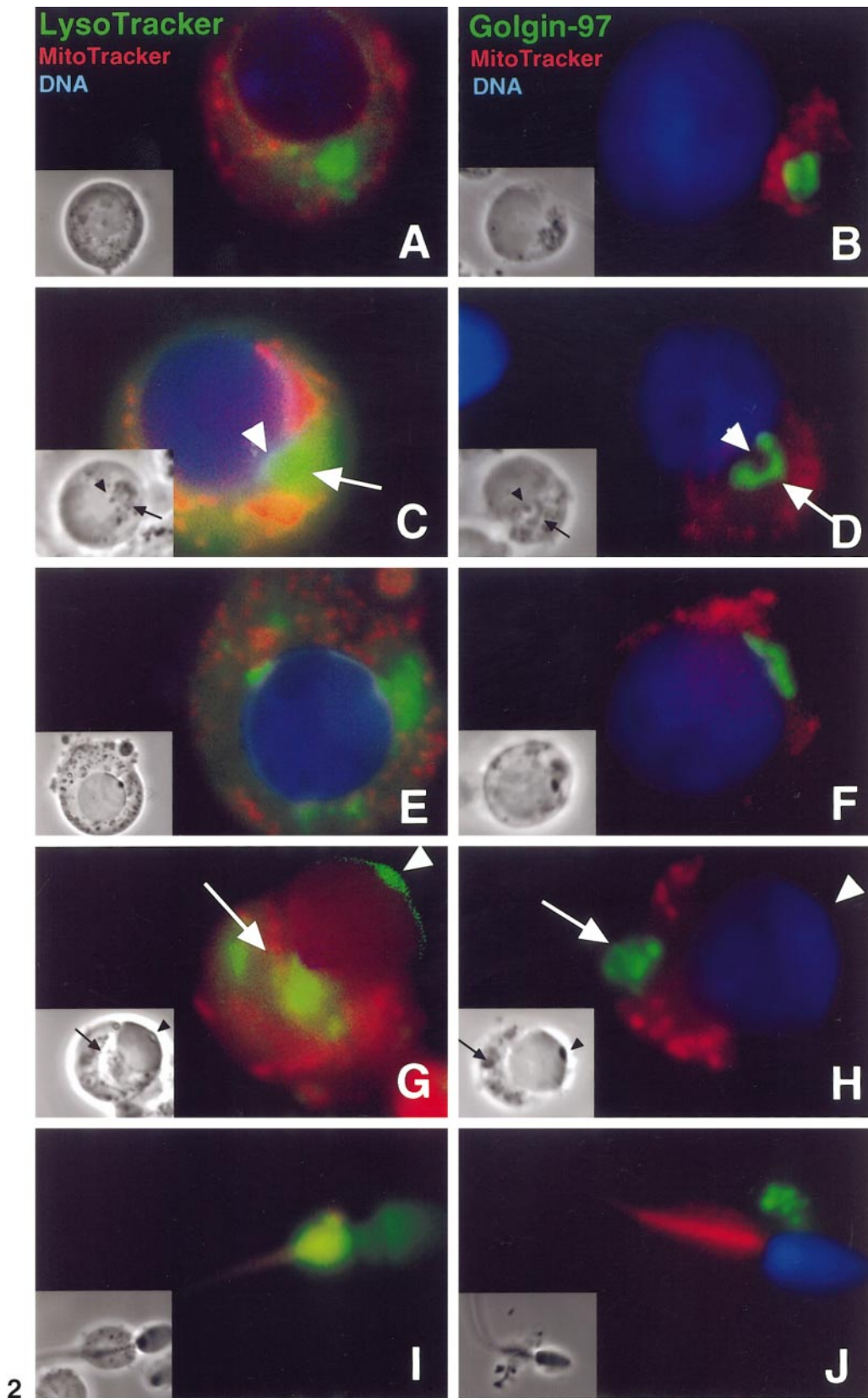
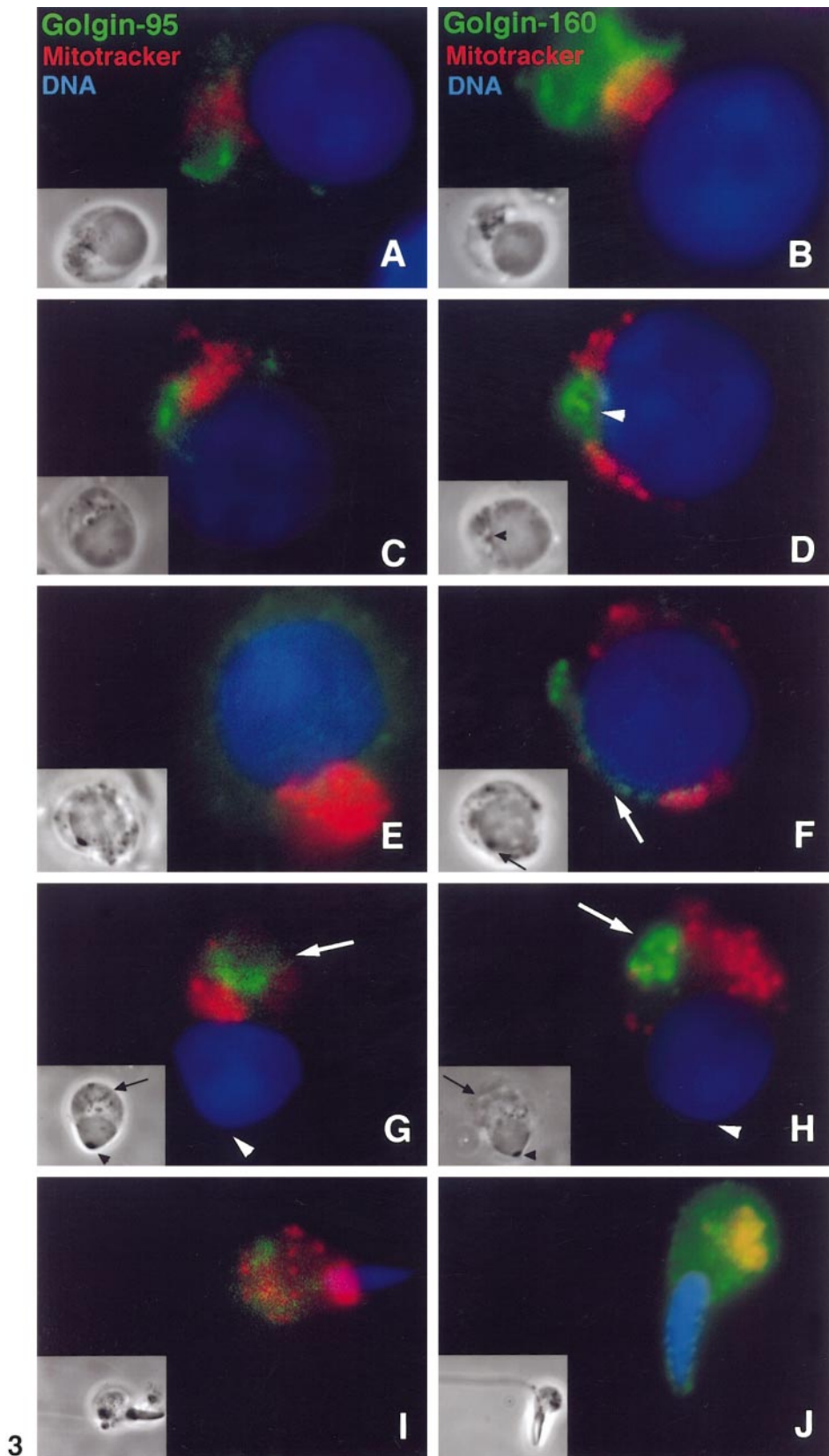


FIG. 1. (A) Ejaculated rhesus spermatozoa were incubated with 1 mM LysoTracker DN-26, washed, and visualized by epifluorescence microscopy. After induction of the acrosome reaction, the acrosomal label disappeared (inset). (B) A mixture of monoclonal antibodies against human acrosin gave a strong label in the acrosomal region of rhesus spermatozoa. (C) This mixture of monoclonal antibodies recognized a strong band of 58 kDa and another weaker band at 31 kDa in an extract of ejaculated spermatozoa (lane 1). After activation of the extract, most of the 58-kDa band disappeared, and there was a concomitant increase in the 31-kDa band, suggesting that the former is proacrosin and the later acrosin (lane 2). (D) The enzyme activity of ejaculated spermatozoa increased significantly after activation for 30 min at pH 8.0. The amount of active enzyme accounted for up to 96% of the total enzyme activity.

FIG. 2. Imaging of the acrosomal vesicle and Golgi apparatus in rhesus monkey spermatids. Acrosomes in living rhesus spermatids (A, C, E, G, and I) were labeled with LysoTracker DN-26 (green) to visualize differentiation. Mitochondria were labeled with MitoTracker CMTMRos (red) and DNA, with Hoechst 33456 (blue). To visualize the Golgi apparatus, living rhesus spermatids were labeled with MitoTracker CMTMRos, fixed, and then stained with the anti-Golgin-97 antibody (green) and DAPI (blue; B, D, F, H, and J). LysoTracker DN-26 was incorporated into the acrosomal vesicle of spermatids since the Golgi phase (C, arrowhead). Besides the acrosomal vesicle, LysoTracker DN-26 labeled another structure that remained in close contact with the acrosomal vesicle and then migrated toward the opposite pole of the cell, along with the mitochondria, in the acrosomal-phase spermatid (C and G, arrow). This structure corresponded to the Golgi apparatus, because Golgin-97 antibodies labeled a structure with a similar position, shape, and behavior during spermatid differentiation (D and H, arrow). Golgin-97 antibodies did not label the acrosomal vesicle or show any sign of cytoplasmic localization in any stage of spermatid differentiation (D and H, arrowhead). Golgin-97 and part of the LysoTracker DN-26 label are incorporated in the cytoplasmic droplet in testicular spermatozoa (I and J).





antibodies resulted in an uniform signal in the same area labeled by LysoTracker DND-26 (Fig. 1B). A Western blot of an acid extract of rhesus spermatozoa reveals a doublet of bands at 58 kDa and a small fraction of a protein at 31 kDa (Fig. 1C, lane 2). After activation, the 58-kDa doublets intensity decreased and the 31-kDa bands intensity increased (Fig. 1C, lane 1). This result suggests that indeed the 58-kDa doublet represents proacrosin and the 31-kDa band represents the activated form of the enzyme. In addition, the activation of the acid extract suggests that 96% of the enzyme is present as proacrosin and 4% is present as acrosin (Fig. 1D).

Imaging the Acrosomal Vesicle, Acrosome Formation, and Mitochondrial Dynamics in Living Rhesus Spermatids

The mitochondrion-specific dye MitoTracker CMTRos detects a distinct subpopulation of mitochondria in either living (Figs. 2A, 2C, 2E, 2G, and 2I) or fixed (Figs. 2B, 2D, 2F, 2H, and 2J) rhesus spermatids. In early round spermatids, the mitochondria are concentrated near the acrosomal vesicle. In elongated spermatids, these organelles are displaced toward the developing flagella (Figs. 2G and 2H) and then reorganized to form the mitochondrial sheath (Figs. 2I and 2J).

Rhesus round spermatids have an acrosomal vesicle that is easy to identify by phase contrast microscopy (Fig. 2C, inset), and LysoTracker DND-26 labels the acrosomal vesicle intensely (Fig. 2C, arrowhead). Consequently, it was possible to follow the flattening and spreading of this organelle over the cell nucleus in later stages of differentiation (Figs. 2E, 2G, and 2I). The LysoTracker DND-26 label remained associated with the acrosome in epididymal spermatozoa (Fig. 2I). This staining pattern was found only in living spermatids, because LysoTracker DND-26 fluorescence is not retained after fixation. LysoTracker DND-26-labeled rhesus spermatids could be cultured up to 6 h in TALP medium without intensity or distribution loss of the label.

LysoTracker DND-26 also labeled a structure close to the acrosomal vesicle and nucleus (Fig. 2C, arrow). This structure has a ribbon-like shape and sometimes seemed to be constituted of several vesicles (Fig. 2E). It was detected in early round spermatids even though the acrosomal vesicle

was not yet present (Fig. 2A). This structure remained in close association with the acrosomal vesicle up to the elongated spermatid stage, when it translocated toward the caudal part of the cell, following the mitochondrial movement (Figs. 2E and 2G, arrow). At this stage it still kept its original shape, but seemed to be subdivided into several layers (Fig. 2G, arrow). Even though this structure was no longer visible in testicular sperm, we found a strong green fluorescence in the cytoplasmic droplet (Fig. 2I) suggesting that this acidic structure was fragmented into smaller vesicles.

Finally, LysoTracker DND-26 could be found in another series of vesicles scattered in the spermatid cytoplasm (Figs. 2A and 2E). Some of these vesicles were in close contact with the nucleus or the acrosome. Because LysoTracker DND-26 labels acidic organelles, these vesicles probably correspond to lysosomes or other intermediate compartments such as endosomes.

Golgi Apparatus Dynamics in Rhesus Spermatids

In order to determine the nature of the structures labeled by LysoTracker DND-26, we decided to use antibodies against known intracellular antigens. Because the ribbon-like structure labeled by the acidotropic probe resembled the Golgi apparatus, three Golgi probes (Golgin-97, Golgin-95/GM130, and Golgin-160) were utilized. These antibodies labeled a horseshoe-shaped structure that faces toward the plasma membrane in early spermatids (Figs. 2B, 3A, and 3B). As the acrosomal vesicle became visible by light microscopy, the Golgi apparatus was over this vesicle (Figs. 2D, 3C, and 3D, arrow). The images obtained with Golgin-97 and -160 are almost identical and suggest that the Golgi apparatus is clamped over the acrosomal vesicle (Figs. 2D and 3D, arrow). It is worthy to note that the images obtained with Golgin antibodies are very similar to the structure labeled by LysoTracker DND-26 at similar stages of differentiation (Figs. 2A and 2C).

As the acrosomal vesicle spread over the nucleus, the intensely focused Golgin-95/GM130 signal disappeared and a diffuse signal in small vesicles scattered through the cytoplasm (Fig. 3E). Golgin-160 looked like beads on a thread and was later found toward the caudal region of the cell (Fig. 3F) with some remnants in the acrosomal area (Fig. 3F, arrow). Golgin-97 spread out, flattened (compare Fig. 2D

FIG. 3. Imaging of the Golgi apparatus in rhesus monkey spermatids. Rhesus spermatids were labeled with MitoTracker CMTRos (red) and then fixed and stained with either the antibody against Golgin-95 (A, C, E, G, and I) or Golgin-160 (B, D, F, H, and J; green). DNA was visualized using the fluorescent dye DAPI (blue). The inset in each picture shows the corresponding phase contrast image. The Golgi apparatus in early Golgi-phase spermatids has a horseshoe-like structure (A and B). As the acrosomal vesicle became evident by light microscopy (C and D, insert), the convex face of the Golgi apparatus faced the cell nucleus (C and D). Later on, the signal of Golgin-95 antibody became cytoplasmic and anti-Golgin-160 started to migrate toward the caudal pole of the cell (E and F). Finally, in elongating spermatids both antigens became concentrated in the same region of the cell (G and H, arrow) and were shed in the cytoplasmic droplet in epididymal sperm (I, J). It is worthy to note that only Golgin-160 appeared to transiently label the acrosomal vesicle in the Golgi stage (F, arrow), but not in later stages (D, G, and H, arrowhead).

with Fig. 2F), and slightly displaced to one side of the acrosomal vesicle. Golgin-97 detection is similar to the juxtannuclear LysoTracker DND-26 pattern, suggesting that both probes are colocalized in or near the same organelle (Fig. 2E). At the elongated spermatid stage, the Golgi apparatus migrated to the caudal pole of the cell, similar to mitochondria (Figs. 2H, 3G, and 3H). Golgin-97 and -160 detected an aggregate of large vesicles (Figs. 2H and 3H, arrow), whereas Golgin-95/GM130 was clustered (Fig. 3G, arrow). None of the Golgi antigens found in the acrosomal vesicle earlier remained there after the organelle trafficking events (Figs. 2H, 3H, and 3G, arrowhead).

The Golgi apparatus was detected in the cytoplasmic droplet of epididymal sperm (Figs. 2J, 3I, and 3J). Golgin-95/GM130 disappeared (Fig. 3I) with only a diffuse label detectable. Golgin-160 scattered into small cytoplasmic vesicles, which were eventually undetectable (Fig. 3H). Golgin-97, however, associated as a big clump of vesicles in the cytoplasmic droplet (Fig. 2J). This association looked very similar to the one found in the previous stage (Fig. 2H). Since the LysoTracker DND-26 label is lost after fixation, any double labelling study in the same cell with anti-Golgin antibodies is not possible. Nonetheless, we think that the detection and dynamics of the Golgi antigens during spermatid differentiation provide evidence that these structures are indeed coincident with the juxtannuclear LysoTracker DND-26 images of the Golgi apparatus.

Localization of Acrosin during Rhesus Spermatogenesis

The punctate pattern of acrosin is surrounded by the spherical Golgi apparatus in spermatocytes (probably in the pachytene stage; Figs. 4A and 4B). These foci probably correspond to proacrosomal granules which, at the round spermatid stage, will fuse and form the acrosomal vesicle (Figs. 4C and 4D). This vesicle can be visualized by phase contrast light microscopy (Fig. 2E, inset) and is always found in close relation to the Golgi apparatus (Figs. 4E and 4F). This physical relationship might have functional consequences: The Golgi-derived membrane vesicles could contribute their contents and remodel to its final shape.

As the acrosomal vesicle and granule flatten, the Golgi apparatus translocates from its apical position to the spermatids caudal pole (Figs. 4G and 4H). In elongated spermatids, the acrosomal matrix spreads over almost half of the nucleus, which has almost reached its final shape (Fig. 2I). Meanwhile, the Golgi apparatus is found in the cytoplasmic droplet with a weak apical signal, perhaps due to fragmentation (Fig. 4I). Epididymal spermatozoa do not contain the Golgi apparatus, and the acrosome is completely formed (Fig. 4J).

Localization of Syntaxin and VAMP in Round Spermatids

Because β -COP (COP I), clathrin-coated vesicles, and rab proteins have been found in the Golgi apparatus of rat

spermatids (Burgos and Gutierrez, 1986; Martinez-Menarguez *et al.*, 1996a,b), other possible components of the intracellular membrane trafficking machinery in rhesus spermatids needed to be investigated. SNAREs are a family of coiled-coil proteins in either a transport vesicle (v-SNARE) or a target membrane (t-SNARE), to which the v-SNARE must deliver its cargo (Nichols and Pelham, 1998). These proteins are involved in endoplasmic reticulum (ER)-Golgi and Golgi-plasma membrane vesicle traffic, and the interaction of specific v- and t-SNAREs triggers fusion between the membranes of a donor vesicle and a target compartment (Hay *et al.*, 1997; Warren and Malhotra, 1998).

The t-SNARE syntaxin is present in the acrosomal vesicle of rhesus spermatids (Fig. 5A). Interestingly, a portion of the label localized in the caudal portion of the cell (Fig. 5A, arrow). This punctate of syntaxin distribution probably corresponds to vesicles involved in trafficking within the Golgi apparatus or from the Golgi to the acrosomal vesicle. The Golgi apparatus is coincident with these syntaxin-containing vesicles (Figs. 2H, 3H, and 4H). The v-SNARE VAMP, however, localized in the acrosomal vesicle, but was not associated with other structures (Fig. 5B). This observation suggests that VAMP might be the syntaxin partner in the fusion of Golgi-derived vesicles with the acrosomal vesicle.

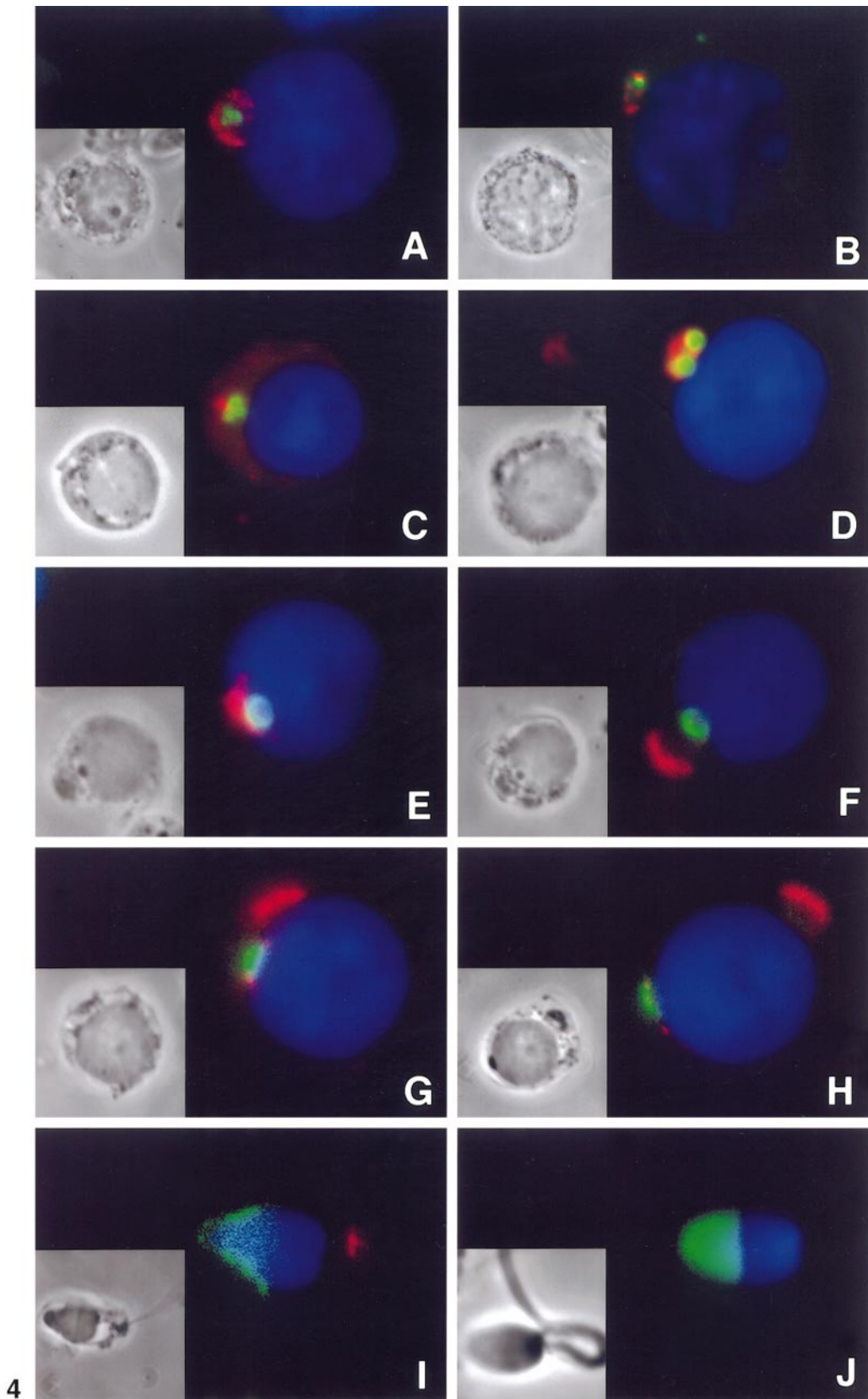
Ejaculated Rhesus Spermatozoa Do Not Contain Golgi Proteins

Imaging of both Golgin-97 (Fig. 6A) and Golgin-95/GM130 (Fig. 6B) did not result in any clear pattern, even though some spermatozoa labeled with anti-Golgin-95/GM130 antibody showed two bands around the equatorial segment (Fig. 6B, arrowhead). Neither of these two antibodies nor the preimmune serum stain <2% of these cells (Figs. 6A and 6B inset). Golgin-160 showed only a faint label on the tail (Fig. 6C, arrow), which was due to nonspecific reactivity with the preimmune serum (Fig. 6C, inset).

Western blot analysis of rhesus testicular cells demonstrated that the antibodies recognized proteins of 160 kDa for Golgin-160, 130 kDa for Golgin-95/GM130, and approximately 97 kDa for Golgin-97, which are the expected molecular weights (Fig. 7, Te). The lower molecular weight bands observed with Golgin-95 and -160 antibodies may correspond to degradation products. However, Golgin-95, -97, or -160 did not show any reactivity with mature sperm (Fig. 7, Sp). The band at 52 kDa in the sperm and testicular extract incubated with Golgin-160 and the one at 41 kDa with the anti Golgin-95/GM130 are nonspecific, because they also appear when the preimmune serum is used (data not shown).

DISCUSSION

The above results demonstrate that LysoTracker DND-26 detects the acrosomes of mature ejaculated rhesus



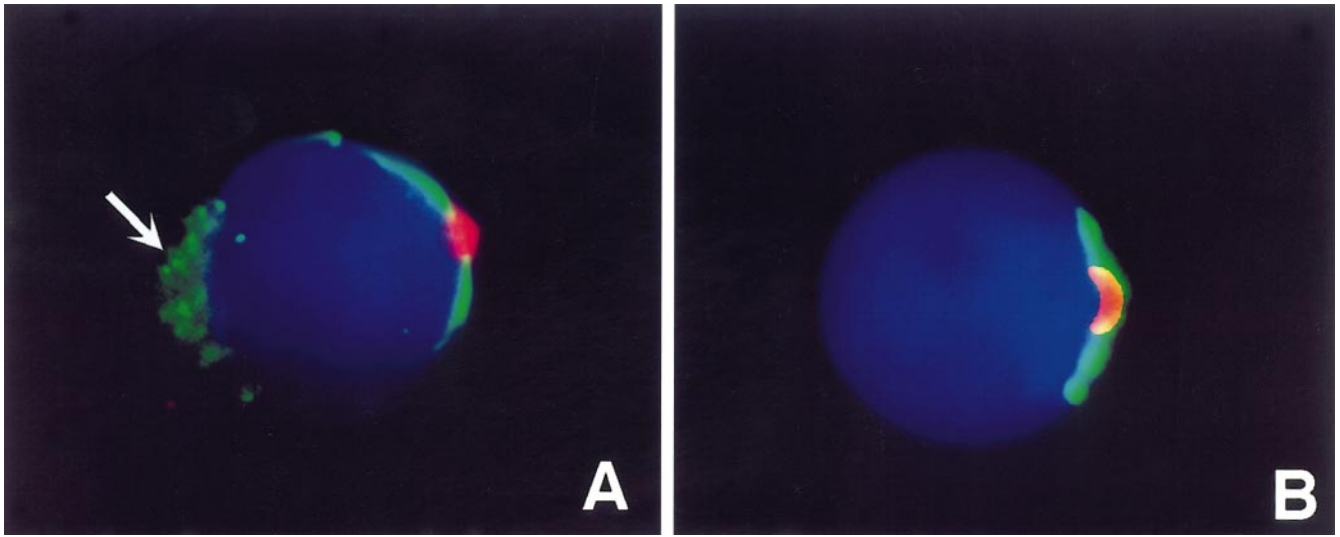


FIG. 5. SNAREs localization in rhesus round spermatids. Fixed rhesus spermatids were stained with a polyclonal antibody against syntaxin or VAMP (B) and a monoclonal antibody against acrosin (red). Syntaxin and VAMP (green) localized in the whole acrosomal vesicle surrounding the acrosomal granule (A, B). Syntaxin also displayed a dotted pattern at the opposite side of the cell (A, arrow) that is probably related to the Golgi apparatus located at that area in this differentiation stage.

spermatozoa. LysoTracker DND-26 also labels the acrosomal vesicle and the Golgi apparatus in live rhesus spermatids. Through imaging, these probes permit investigators to study the development of the acrosome and Golgi dynamics in living cells.

Acrosomal Vesicle Formation and Sorting of Proacrosomal Granules to the Perinuclear Theca in Spermatids: Role of Intraluminal pH and SNAREs Proteins

Because the acrosome is formed by the coalescence of Golgi-derived vesicles, and a large number of acrosomal proteins are proteases, it has been postulated that the acrosome is a modified lysosome (Hartree, 1975). However, lysosomal (lgp-120) or late endosomal (cation-dependent and -independent mannose 6-phosphate receptor) markers appear to be associated with lysosomes in rat spermatocytes, but not with the acrosome or proacrosomal vesicles

(Martinez-Menarguez *et al.*, 1996a). Therefore, the lysosomal paradigm for acrosome biogenesis may need revisiting.

In the secretory pathway, posttranslational processing of secretory proteins and cleavage of prohormones is pH-dependent (Orci *et al.*, 1986, 1994; Colomer *et al.*, 1996). Delivery of pH-sensitive probes by pinocytosis or receptor-mediated internalization has facilitated the study of the pH along the endocytic pathway, revealing the acidity of the lumen increases progressively, from a pH of 6.5 to 6.0 in early and recycling endosomes to 4.5 in lysosomes (Llopis *et al.*, 1998). Conversely, the pH inside subcompartments of the secretory pathways is thought to be the highest in the endoplasmic reticulum, becoming more acid as the secretory products approach the plasma membrane (Demaurex *et al.*, 1998). Secretory granules can attain a pH as low as 5.5 (Orci *et al.*, 1994; Demaurex *et al.*, 1998). We show here that the acidothrophic probe LysoTracker DND-26 labels the Golgi apparatus of rhesus spermatids. Probably the probe label the trans-Golgi and/or the trans Golgi network

FIG. 4. Immunolocalization of acrosin and Golgi apparatus during rhesus spermatid differentiation. Rhesus spermatids were mounted onto poly-L-lysine-coated coverslips, fixed, and stained with antibodies against acrosin (green), a mixture of anti-Golgin-160 and Golgin-97 (red), and DAPI for DNA (blue). At the spermatocyte stage, acrosin displayed a dotted pattern surrounded by the Golgi apparatus with a spherical shape (A, B). In Golgi-phase spermatids the acrosin granules started to fuse to each other, forming the acrosomal vesicle (C-F). The Golgi apparatus displayed a horseshoe-like shape with the convex side facing the acrosomal vesicle (C-F). Later on, the acrosomal vesicle and granule began to flatten, and the Golgi apparatus migrated toward the opposite pole of the cell (G, H). In the latest stage of differentiation (probably maturation phase), acrosin had reached almost half of the spermatid nucleus surface and there were only some traces of the Golgi apparatus in the residual body (I). Finally, in a mature epididymal sperm, there was no trace of Golgi antigens, and the acrosome had reached its final shape (J).

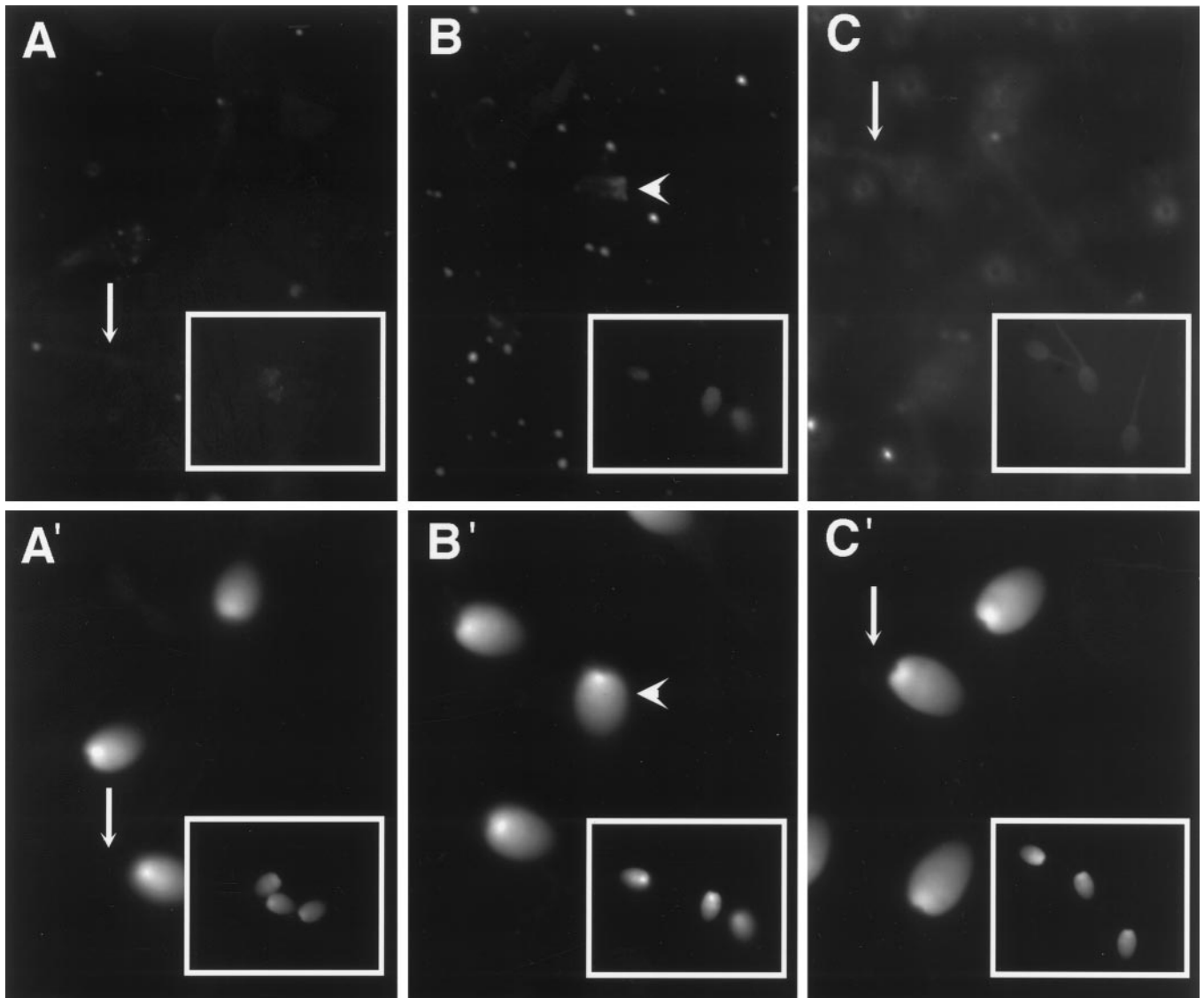


FIG. 6. Mature ejaculated rhesus spermatozoa do not have Golgi antigens. Ejaculated rhesus sperm were mounted onto poly-L-lysine coverslips and stained with antibodies against Golgin-97 (A), Golgin-95/GM130 (B), or Golgin-160 (C). Some of them show a faint strip (arrow) or a double strip (arrowhead) in the head region. The inset shows staining with the preimmune serum. The bottom row (A', B', and C') corresponds to the same sperms stained with the DNA dye DAPI.

(TGN), because these are the most acid subcompartments of the organelle in somatic cells. LysoTracker DND-26 has been used to label lysosomes in somatic cells. Since the lysosomes have a significantly lower pH than the Golgi apparatus, it is possible that the label of the Golgi had been obscured by the more intense contribution of lysosomes. Alternatively, the Golgi apparatus of spermatids has a lower pH than somatic cells in a way that allows a higher uptake of the dye. The latter explanation would reflex a significant difference between spermatogenic and somatic cells (De-maurex *et al.*, 1998; Llopis *et al.*, 1998). LysoTracker

DND-26 fluorescence is detected at the earliest stages of acrosome formation. However, this acidification might occur in the proacrosomal granules, in the acrosomal vesicle itself, or in both structures. The fluorescence intensity of LysoTracker DND-26 does not change markedly at different stages of development. What is the function of the acidic intraluminal space of the acrosomal vesicle? The round spermatid is a polarized cell engaged in the formation of a highly regulated secretory granule. Perhaps the acidic intraluminal pH of the acrosomal vesicle (and probably of proacrosomal granules) aids in the compartmentalization

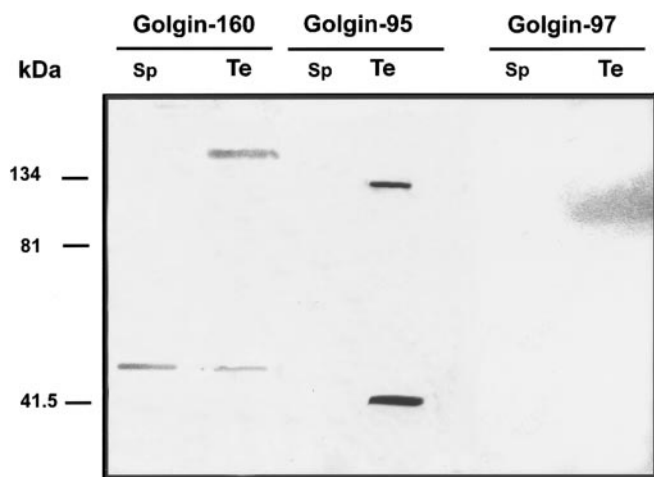


FIG. 7. Characterization of Golgin-95, -97, and -160 in rhesus monkey spermatozoa and testis. Extracts of ejaculated rhesus sperm (Sp) and rhesus testis (Te) were loaded onto a 10% SDS-PAGE under denaturant and reducing conditions. These proteins were blotted onto a nitrocellulose membrane and probed with antibodies against Golgin-160, Golgin-95, and Golgin-97. The three antibodies reacted to proteins of the expected molecular weights in the testicular extract: 160, 130, and 97 kDa, respectively. Golgin-160 and -95 also reacted with other proteins of lower molecular weight that probably correspond to partial degradation. Neither Golgin-95 nor Golgin-97 antibodies reacted with any protein in rhesus sperm extract. The two low-molecular-weight bands appearing with anti-Golgin-160 correspond to nonspecific staining, because they also appear using the preimmune serum (data not shown).

and sorting of acrosomal proteins, as postulated for neuroendocrine secretory vesicles (Carnell and Moore, 1994; Tooze, 1998). Proacrosomal granules may therefore be analogous to immature secretory granules (ISGs). One proposed mechanism for sorting proteins into secretory granules is via selective aggregation, triggered by low pH and high calcium concentration (Kuliawat and Arvan, 1994; Carnell and Moore, 1994; Kuliawat *et al.*, 1997). The aggregation process may be driven either by the sorted proteins or by helpers such as the members of the granin family (Natori and Huttner, 1996). In this regard it is interesting to note that Golgin-97 has a domain homologue to human granin (Griffith *et al.*, 1997). In the acrosomal vesicle, the low intraluminal pH may play a role in the packing and compartmentalization of acrosomal proteins, and misrouted soluble proteins may be retrieved from the acrosome by a clathrin-mediated pathway (Martinez-Menarguez *et al.*, 1996b).

Proacrosomal granule fusion and other trafficking events important for acrosome maturation probably involve the NSF-SNAP (soluble NSF attachment protein)-SNARE machinery for targeting, docking, and fusion of intracellular membranes (Ungerman *et al.*, 1998; Nichols and Pelham, 1998). If correct, this model predicts that fusion between

two membranes takes place following the interaction of an integral component in the plasma membrane of the v-SNARE and a complement component on the t-SNARE. The reaction also requires SNAPs. The formation, fusion, and intracellular traffic of proacrosomal granules probably also involves similar kinds of proteins. In fact, syntaxin, SNAP-25 (t-SNAREs), and VAMP (v-SNARE) are expressed in sea urchin sperm. Their association with the plasma membrane increases following the acrosome reaction, and they are shed from the sperm head in small vesicles (Schulz *et al.*, 1998). Therefore, it seems that the same fusion mechanism governing secretory granule exocytosis in somatic cells is also active during acrosome reaction secretion.

In this context, during early Golgi-phase round spermatids, proacrosomal granules containing either VAMP or syntaxin will start to fuse, forming the acrosomal granule. Because these granules are formed by pachytene spermatocytes, these cells must have a mechanism to prevent a precocious fusion of these vesicles. Whether the positioning of the acrosomal vesicle to the perinuclear theca also requires the participation of SNAREs remains to be determined. Syntaxin and VAMP can be detected in the acrosomal vesicle either by indirect immunofluorescence (Fig. 6) or by immunoelectron microscopy (data not shown). Because syntaxin is also localized near the Golgi in round spermatids, perhaps this fusion protein is also involved in intracellular vesicular trafficking to another cell compartment beside the acrosomal vesicle. VAMP's presence in the acrosomal vesicle may be a remnant of the fusion process between proacrosomal granules or it may have a function in later stages of acrosome biogenesis (Schulz *et al.*, 1998).

These results further indicate that LysoTracker DND-26 is a good noninvasive probe to label acidic organelles in mammalian spermatids. In addition to the acrosomal vesicle and Golgi apparatus, other vesicles are detected by LysoTracker DND-26 (Fig. 2). These vesicles probably correspond to lysosomes, endocytic vesicles, or another acidic vesicle involved in intracellular trafficking. LysoTracker DND-26 did not label the ER as confirmed by immunocalcification of the resident protein calreticulin (data not shown). Therefore, LysoTracker DND-26 along with the probes MitoTracker CMTRos and Hoechst 33344 are useful to dynamically visualize the acrosomal vesicle, the Golgi apparatus, the mitochondria, and the cell nucleus in living spermatids.

Dynamics of the Golgi Apparatus in Rhesus Spermatids

The relative contribution of Golgi-, ER-, acrosome-, and plasma-membrane-derived vesicular transport and molecular mechanism during spermiogenesis can be proposed. In spermatocytes, the Golgi apparatus is an eccentric sphere surrounding the proacrosomal granules (Figs. 4A and 4B). In round spermatids, the Golgi apparatus is crescent shaped,

facing the plasma membrane in a cis–trans configuration (Figs. 4C and 4D).

Why does the Golgi apparatus adopt this new configuration in round spermatids? The answer might be related to the transformation from a nonpolarized cell (spermatocyte) to a polarized one (round spermatid) and to the establishment of a new pattern of vesicular trafficking to build different cytoplasm and plasma membrane domains in a mature spermatozoon. Moreover, in early–mid round spermatid differentiation, the Golgi apparatus changes its orientation, becoming a cis–trans configuration facing the nucleus. This new position puts the trans-Golgi in close proximity to the nucleus and the cytoskeletal perinuclear theca that circumscribes the nuclear envelope. Perhaps this orientation assures a direct vesicular flow from the Golgi apparatus to the acrosomal vesicle. The molecular mechanism used by round spermatids for acrosome biogenesis and the different plasma membrane domains might involve signals and determinants that are active in other polarized and/or secretory cells (reviewed by Keller and Simons, 1997). Other proteins probably involved in intracellular traffic in round spermatids include rab-related proteins, COPs, and clathrin. β -COP-containing coated vesicles (COP I vesicles) are present in the cis, medial, and trans side of the Golgi apparatus in rat round spermatids (Martinez-Menarguez *et al.*, 1996b; West and Willison, 1996). Three Golgi apparatus probes localized this organelle to a similar region in rhesus spermatids, though each has specific localization patterns in cultured cells. Golgin-97 localizes in the trans-Golgi (Lu *et al.*, 1998) and Golgin-95/GM130 localizes at cis (Nakamura *et al.*, 1995). Golgin-95 and GM130, identified independently, correspond to the same protein (Golgin-95/GM130). GM130 seems to be part of the β -COP-coated vesicles during anchoring to the Golgi membranes along with p155 (Nakamura *et al.*, 1995, 1997).

In cultured cells, double-immunofluorescence microscopy has given a striking illustration of the close association of the Golgi elements with the centrosome and how the overall organization is changed after drug-induced depolymerization of microtubules (Ho *et al.*, 1989; Cole *et al.*, 1996). There are a few studies about the structure of the microtubule-based cytoskeleton in mammalian spermatids and its relation to the position and functional state of the Golgi apparatus. In early round spermatids, the Golgi apparatus appears to be close to the centrioles (de Kretser, 1969). Perhaps the migration of the Golgi apparatus to the caudal pole of the spermatid uses microtubule motor proteins, especially plus-end-directed kinesin (Lippincot-Schwartz *et al.*, 1995; Vaisberg *et al.*, 1996). Both kinesin and dynein are concentrated in the sperm manchette, a circular array of microtubules surrounding the nucleus, and there are physical connections between vesicles and microtubules in the manchette (Fawcett *et al.*, 1971; Hall *et al.*, 1992; Miller *et al.*, 1999). Even though the polarity of the manchette microtubules is not yet known, an electron microscopy study has shown periodic densities in the nuclear ring, possibly organizing a microtubule organizing center

(MTOC) foci (Russell *et al.*, 1991). The fate of the manchette is unclear, but it seems that it is eventually incorporated in the residual body along with the Golgi apparatus, the ER, and the chromatoid body. The mechanism of Golgi migration during cell differentiation may be similar to that used during mitosis, when this organelle is segregated into the two daughter cells (Shima *et al.*, 1998).

Direct injection of sperm into mature oocytes (intracytoplasmic sperm injection) has become a popular technique to produce mice, rhesus, and human offspring (Palermo *et al.*, 1992; Kimura and Yanaguimachi, 1995; Hewitson *et al.*, 1999). Moreover, intracytoplasmic injection of elongated spermatids (ELSI) and even round spermatids (ROSI) in rabbits, mice, and humans has been reported to yield viable offspring albeit at exceedingly low rates (Sofikitis *et al.*, 1994; Tesarik *et al.*, 1995; Sasagawa *et al.*, 1998); however, these publications have yet to be confirmed. Our results show that the position and likely functional status of the Golgi apparatus changes depending upon the spermatid differentiation step. Hence, the injection of a fully active Golgi apparatus might have unexpected consequences in vesicular dynamics and sorting of the different vesicles en route to the developing acrosomal vesicle. This behavior may have important implications in the usage of spermatogenic cells for assisted reproductive technology.

In summary, our results indicate that the acrosome is an acidic compartment from its very early stages of development, as is the Golgi apparatus. During differentiation, the close physical relationship between the acrosome and the Golgi apparatus is lost as the Golgi is translocated toward the opposite pole of cell. At this end, the Golgi apparatus is displaced to the cytoplasmic droplet. These observations were confirmed in living cells using antibodies specifically directed against Golgi proteins and acrosomes. Studies are needed to further elucidate the molecular basis of the migratory behavior of the Golgi apparatus and its functional state during the different stages of spermatid differentiation.

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