Introduction

The acrosome is a specialized membrane-bound vesicle located on the head of spermatozoa. It contains hydrolytic enzymes that are exocytosed during the ‘acrosome reaction’, the membrane fusion event that is a prerequisite for sperm penetration through the zona pellucida and is crucial also for subsequent sperm–egg fusion. Electron microscopy and cytochemical studies have indicated the involvement of the Golgi apparatus in acrosome formation (Susi et al., 1971; Tang et al., 1982). Proacrosomal vesicles from the trans-Golgi network (TGN) in the round spermatid fuse with one another to produce an acrosomal vesicle. The membrane of the acrosomal vesicle then extends as a bilaminar structure covering the anterior hemisphere of the nucleus of the spermatid. The materials for the developing acrosome seem to be supplied by Golgi-derived coated vesicles and an acrosomal vesicle, and finally became distributed as multiple small units over the whole surface of an acrosomal cap in the round spermatid. The mode of acrosome formation in musk shrews was distinctly different from that in rats and mice, in which the Golgi apparatus remains as a single unit throughout acrosome formation. In musk shrews, the proacrosomal vesicles formed successively by the Golgi satellites coalesced, one after another, into a potential acrosomal vesicle. This process may result in further enlargement of the acrosome. The results of the present study indicate that Golgi satellites are necessary for the biogenesis and development of the giant acrosome in musk shrew spermatozoa.
Materials and Methods

Animals

Testes were removed surgically from ether-anaesthetized adult male musk shrews. The animals were either born and reared at Kyushu University or came from the Laboratory of Animal Management, School of Agricultural Sciences, Nagoya University.

Antibodies and immunoblot analysis

Antibodies against both Rab6p and p58 were used to immunolabel the Golgi apparatus. Rab6p is a small GTPase associated mainly with the Golgi stacked cisternae (Goud et al., 1990). The synthetic peptide used for raising antibody is derived from the COOH-terminal hypervariable region (PGMESTQDRSREDMID) of human Rab6p (Zahraoui et al., 1989). The antibody against Rab6p recognizes a protein migrating at 25 kDa on a blot (Iida et al., 1997, 1999). Monoclonal antibody against p58 was obtained from Sigma Chemical Co. (St Louis, MO). p58 is a microtubule-binding peripheral Golgi membrane protein (Bloom and Brashear, 1989).

Seminiferous tubules were homogenized in PBS and centrifuged at 1000 g for 15 min to remove the nuclear fraction. The supernatant was centrifuged at 100 000 g for 60 min to collect the membrane fraction for electrophoresis. Proteins of the samples dissolved in electrophoresis sample buffer were separated on SDS-PAGE and transferred to nitrocellulose sheets. The sheets were incubated for 2 h with the anti-Rab6 antibody diluted 1:1000 with a blocking buffer (PBS containing 5% (w/v) non-fat milk and 0.1% (v/v) Tween-20) and then incubated with goat anti-rabbit IgG conjugated with horseradish peroxide (BioRad, Richmond, CA) diluted 1:2000 in the same buffer. Antigen-antibody complexes were visualized using an enhanced chemiluminescence (ECL) detection kit (Amersham Pharmacia, Piscataway, NJ) as described by Iida et al. (1996).

Immunocytochemistry

Testis tissues, fixed by immersion in 3% (w/v) paraformaldehyde dissolved in PBS, were frozen in liquid nitrogen after infiltration of 2.3 mol sucrose l^{-1}. Cryosections of the testis (8 μm) were attached to poly-L-lysine-coated glass slides, treated for 15 min with 50 mmol NH4Cl l–1 in PBS, and washed in PBS three times. The samples were incubated with the anti-Rab6 antibody and then incubated with Texas red-conjugated anti-rabbit Ig (Amersham Pharmacia). The samples were stained with the anti-Rab6 antibody and the monoclonal anti-p58 antibody and then incubated with a mixture of Texas Red-conjugated anti-rabbit Ig and FITC-conjugated anti-mouse Ig (Amersham Pharmacia) for double labelling. In controls, the primary antibody was replaced by pre-immune serum IgG. Stained samples were examined either with a fluorescence microscope (Olympus BX-50) or with a confocal laser scanning microscope (Olympus LSM-GB 200).

Electron microscopy and light microscopy

Samples for electron microscopy were fixed by immersion in 3% (v/v) glutaraldehyde in 0.1 mol cacodylate buffer l^{-1} (pH 7.4), postfixed in 1% (v/v) osmium tetroxide, dehydrated in a graded series of ethanol and embedded in epoxy resin. Ultrathin sections were examined under a Hitachi H-100 electron microscope after staining with uranyl acetate and lead citrate. Thick sections were examined under a light microscope after staining with toluidine blue. The musk shrew spermatozoa released from the cauda epididymidis were visualized under a microscope (Olympus BX-50) equipped with differential interference contrast (DIC) optics.
Results

Immunoblot analysis

Immunoblot analysis showed that the anti-Rab6 antibodies, raised against the synthetic peptide of human Rab6 (Iida et al., 1997), recognized a single band migrating at 25 kDa on a blot to which the total membrane proteins of seminiferous tubules of the musk shrew testis had been transferred (Fig. 1). The specific antibody against Rab6p was used for immunocytochemistry to examine the localization of the Golgi apparatus.

Light microscopy

The cycle of the seminiferous epithelium in musk shrews has 13 stages, and spermatid development is subdivided into 13 steps (Kurohmaru et al., 1994). The enormous fan-shaped acrosome seen in mature spermatozoa (Fig. 2) is completely formed by step 13. In the present study, early acrosomal development is divided into three phases for convenience. During the early Golgi phase, few proacrosomal vesicles were visible in spermatids under a light microscope (step 1, Fig. 3a). The period from the appearance of proacrosomal vesicles to the development of a hemispherical acrosomal vesicle fixed to the spermatid nucleus is referred to as the late Golgi phase (steps 2–4, Fig. 3b). During the cap phase, a developing acrosome surrounded the nucleus in spermatids (step 5, Fig. 3c).

Confocal laser scanning microscopy

The localization of the Golgi apparatus in round spermatids during the cap phase was demonstrated by immunocytochemistry. Confocal laser scanning microscopy on cryosections of musk shrew testis revealed that immunostaining with the anti-Rab6 antibody produced peculiar fluorescence spots that were hemispherically dispersed in round spermatids (Fig. 4a,b). A similar immunostaining pattern was observed when the specimens were stained with the anti-p58 antibody (data not shown). Merging the immunofluorescence spots with the nucleus of the spermatid (Fig. 4c) indicated that the spots probably represented the isolated Golgi apparatus located in association with the surface of the developing acrosome, and that the space between the nucleus and the fluorescence spots corresponds to the acrosomal cap (Fig. 4c). No specific labelling was observed when the anti-Rab6 antibody was replaced by preimmune serum (data not shown). Double immunostaining with the anti-Rab6p antibody and the monoclonal anti-p58 antibody revealed that p58 immunofluorescence labelling was always associated with Rab6p immunofluorescence labelling (Fig. 4d), indicating that the fluorescence spots represent the isolated Golgi apparatus associated with the developing acrosome. However, there was little overlap of the two antigens on the isolated Golgi apparatus (Fig. 4d, right panel), indicating that they were situated on separate loci within the organelle.

The three-dimensional distribution of the isolated Golgi apparatus on the surface of the developing acrosome was examined using confocal laser scanning microscopy by focusing up or down stepwise within the 8 µm cryosections immunostained with the anti-Rab6 antibody (Fig. 5a–d). The pattern of fluorescence spots indicated that much of the isolated Golgi apparatus (the ‘Golgi satellites’) is distributed over the whole surface of the developing acrosome.

Cryosections of rat testis were immunostained with the anti-Rab6 antibody to compare the distribution and the construction of the rat Golgi apparatus in the spermatids with that of musk shrews. Rab6 immunofluorescence labelling representing the Golgi apparatus appeared as a single spot in the juxtanuclear cytoplasm in all cells of rat.
Fig. 4. Confocal laser scanning microscope images of the distribution of the isolated Golgi apparatus in spermatids of the musk shrew, Suncus murinus, at low (a) and high magnification (b–d). Samples are either stained by the anti-Rab6 antibody (a–c) or double-stained by both the anti-Rab6 antibody and the anti-p58 monoclonal antibody (d). In (c) a nuclear image of a round spermatid is visualized by over-enhancement of a laser beam (green), and the image is merged with Rab6 immunofluorescence labelling (red). In (d) a spermatid is double-stained with the anti-p58 monoclonal antibody (left) and the anti-Rab6 antibody (centre). A confocal image produced by superposition of the left (green) and centre (red) panels is shown in the right panel. N, nucleus. Asterisks indicate an unstained acrosomal cap. Scale bars represent 10 (a), 5 (b,d) and 3 μm (c).
Golgi satellites in musk shrew spermatids

Fig. 5. Confocal laser scanning microscopy showing the three-dimensional distribution of Golgi satellites immunostained with the anti-Rab6 antibody in round spermatids of the musk shrew, Suncus murinus. Four confocal images are obtained by changing the focal plane stepwise in 8 μm cryosection from the bottom (a) to the top (d). The arrow indicates the same spermatid throughout the micrographs. Compare the hemispherically disposed fluorescence in (b) with the spotted fluorescence in (d). Scale bar represents 3 μm.

Fig. 6. (a) Confocal laser scanning microscope images showing the distribution of Golgi apparatus stained with the anti-Rab6 antibody in a rat seminiferous tubule. In (b), the nuclei of mature spermatozoa are visualized by over-enhancement of a laser beam (green), which is merged with Rab6 immunofluorescence labelling (red). Note that labelled Golgi in round spermatids appears as a single spot. Scale bar represents 10 μm.
seminiferous epithelium (Fig. 6), which is in marked contrast to the immunostaining pattern in musk shrew testis (Figs 4 and 5). An immunostaining pattern similar to that of rat testis was also observed in mouse testis (data not shown).

The distribution of the Golgi satellites of musk shrew spermatids in the early and late Golgi phases were examined. Under the light microscope, proacrosomal vesicles and an acrosomal vesicle were not visible in the early Golgi phase, but were detectable in the late Golgi phase (Fig. 3). Confocal laser scanning microscopy revealed that Rab6 immunofluorescence labelling appeared as a large aggregate localized in the vicinity of the nuclei of spermatids in the early Golgi phase (Fig. 7a,b). In the late Golgi phase, the Golgi satellites stained with the anti-Rab6 antibody were distributed alongside or between proacrosomal vesicles and an acrosomal vesicle adherent to the nucleus (Fig. 7c–e).

**Electron microscopy**

The structure of the Golgi satellites of round spermatids in both the late Golgi phase (Fig. 8) and the cap phase (Fig. 9) were examined under an electron microscope. In the late Golgi phase, several proacrosomal vesicles were observed in...
the vicinity of an acrosomal vesicle adherent to the nuclear envelope (Fig. 8). The proacrosomal vesicles ranged from 1 to 3 μm in diameter. The Golgi satellites and mitochondria were associated with proacrosomal vesicles and an acrosomal vesicle.

During the cap phase, the Golgi satellites were localized to the vicinity of the surface of the developing acrosome (Fig. 9a). The individual Golgi satellites appeared to be segregated from one another, and a membranous connection between them was not observed. The size of the individual Golgi satellites (1-2 μm) was smaller than that of the rat spermatid Golgi apparatus (4-6 μm; Susi et al., 1971). The Golgi satellites were composed of several stacked cisternae and a peculiar aggregation of many small vesicles (Fig. 9b). The small vesicles tended to accumulate in the concave space of the stacked cisternae of the Golgi satellites (Fig. 9a and b). Golgi cisterna-like tubular structures were occasionally intermingled with the vesicles (Fig. 9c).

Discussion

During formation of the mammalian acrosome, proacrosomal vesicles are formed at the concave face of the Golgi apparatus. Several proacrosomal vesicles coalesce into a single, large acrosomal vesicle, which adheres to the surface of the nuclear envelope. In general, further proacrosomal vesicles are not formed after the formation of a nucleus-adherent acrosomal vesicle. The acrosomal vesicle then spreads over the anterior hemisphere of a nucleus in the form of a cap. In the
Fig. 9. Electron micrographs showing Golgi satellites in the spermatid of the musk shrew, Suncus murinus, in the cap phase. (a) The developing acrosome is associated with the Golgi satellites (arrows). Note many small vesicles associated with the Golgi satellites (b,c). The arrow in (c) indicates a Golgi cisterna-like tubular structure. AC, acrosomal cap. Scale bars represent 1 (a), 0.5 (b) and 0.4 μm (c).
spermatids of most mammals, usually only one Golgi apparatus remains closely associated with the developing acrosome throughout acrosomal formation during both the Golgi phase and the Cap phase (Susi et al., 1971; Burgos and Gutierrez, 1986). This finding is consistent with the observation in the present study that Rab6 immunofluorescence labelling representing the Golgi apparatus appeared as a single spot in the rat spermatid.

The mode of acrosome formation in musk shrew spermatids deviated from that in most other mammals in several ways. First, the outer acrosomal membrane of the acrosomal cap was associated with multiple small Golgi units, the Golgi satellites, distributed over the whole surface of the developing acrosome during the cap phase. This finding is consistent with the result obtained by electron microscopy. Association of multiple, but not single, Golgi satellites with the acrosomal cap probably reflects an enormous requirement for material during the development and maturation of the giant acrosome.

Second, the Golgi satellites in musk shrew spermatids changed their architecture during acrosome formation. In the early Golgi phase, in which few proacrosomal vesicles are present, the Golgi satellites aggregated in the juxtanuclear cytoplasm. As acrosome formation progressed from the early Golgi phase to the late Golgi phase, the Golgi satellites gradually dispersed and became associated with proacrosomal vesicles and an acrosomal vesicle, and were finally distributed over the whole surface of the acrosomal cap covering the anterior half of the nucleus. These observations indicate that the Golgi satellites in round spermatids markedly change their three dimensional configuration as acrosomal formation progresses, a phenomenon which is a characteristic of acrosomal formation in musk shrews but which has not been reported in spermatids of other mammals.

Third, large proacrosomal vesicles in musk shrew spermatids were still produced successively after formation of a nucleus-adherent acrosomal vesicle. Close association of the Golgi satellites with the proacrosomal vesicles indicates the involvement of the Golgi satellites in their production. The proacrosomal vesicles seemed to coalesce one after another into an acrosomal vesicle, which may have resulted in further enlargement of the acrosome. This finding indicates that contributions from the ‘active’ Golgi satellites are required throughout the Golgi phase for the successive production of proacrosomal vesicles and the subsequent enlargement of the acrosome.

Since acrosomal formation is accomplished by successive vesicle transport, the process seems likely to be regulated by small GTPase Rab proteins, the regulators of intracellular membrane traffic (Pfeffer, 1992; Zerial and Stenmark, 1993; Gonzalez and Scheller, 1999). Among more than 35 Rab proteins, a Rab6 GTPase appears to be a leading candidate for regulating acrosome formation because of its involvement in the formation of post-Golgi vesicles in addition to its regulation of intra-Golgi vesicular traffic (Jones et al., 1993; Iida et al., 1997). However, in the present study, Rab6p was not detected on the acrosome of rat spermatozoaa (Iida et al., 1999) or the developing musk shrew acrosome, indicating that Rab6p is not directly involved in acrosome formation. It is possible that other Rab proteins are involved in the membrane traffic responsible for acrosome formation, as three or more small GTPases have been reported to be present in spermatozoa (Ward et al., 1999).

In conclusion, the results of the present study indicate that the mode of acrosome formation in musk shrews differs from that in the spermatids of most other mammals, in that the Golgi apparatus in musk shrew spermatids functions in multiple small units, Golgi satellites, involved in the successive formation of proacrosomal vesicles, which coalesce into a nucleus-adherent acrosomal vesicle, resulting in formation of a giant acrosome.

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