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INTRATESTICULAR DISTRIBUTION OF CYRITESTIN, A PROTEIN INVOLVED IN GAMETE INTERACTION

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Summary

Cyritestin, a member of the ADAM family of proteins, has been shown to be involved in the interaction between sperm and egg during fertilization. The protein is a transmembrane protein associated with the sperm acrosome. In the present study, electron microscopy was used to trace the distribution of the cyritestin molecule in intratesticular germ cells, particularly in haploid round spermatids where the acrosomal structure is differentiating. Our results indicate that cyritestin is transported to the forming acrosomal vesicle through the Golgi apparatus to become part of the acrosomal membrane. Differential staining with antibodies recognizing either the metalloprotease-like domain or the cytoplasmic domain of cyritestin indicates that processing of the molecule leading to the loss of the pro- and metalloproteinase-like domains begins during germ cell stage 6 and is completed before stage 15.

Key words: ADAM proteins, germ cell development, acrosome, protein processing, intracellular transport.

Introduction

The members of the ADAM (*a d*isintegrin *a*nd *m*etalloprotease) family of proteins are characterized by the presence of metalloprotease-like, disintegrin-like and epidermal growth factor (EGF)-like domains (see Perry *et al.* 1992; Blobel *et al.* 1992; Wolfsberg *et al.* 1993, 1995*b*; Heinlein *et al.* 1994; Jia *et al.* 1996). A considerable number of these proteins are transmembrane proteins involved in specialized cellular interactions such as gamete and myoblast fusion (Myles *et al.* 1994; Evans *et al.* 1995*b*; Yagami-Hiromasa *et al.* 1995; Linder and Heinlein, 1997), cellular recognition phenomena during embryonic development (Alfandari *et al.* 1997) and neurogenesis (Rooke *et al.* 1996) and in the processing of biologically active factors (Lunn *et al.* 1997; Moss *et al.* 1997). Data from other studies indicate further roles for ADAM proteins in a variety of organisms (Emi *et al.* 1993; Wolfsberg *et al.* 1995*a*; Podbilewicz, 1996; Howard *et al.* 1996; Jia *et al.* 1996; Weskamp *et al.* 1996; Kuno *et al.* 1997; McKie *et al.* 1997; Shilling *et al.* 1997).

One member of the ADAM family of proteins is cyritestin (Heinlein *et al.* 1994), a transmembrane protein of the acrosomal membrane (Linder *et al.* 1995). The gene for cyritestin, *Cyrn1*, is found on mouse chromosome 8 and is exclusively expressed in testicular germ cells (Lemaire *et al.* 1994), giving rise to a 110 kDa precursor protein that is processed to the 55 kDa form during the late stages of sperm differentiation (Linder *et al.* 1995). After sperm interaction with the zona pellucida and a successful acrosome reaction, cyritestin becomes distributed over the entire sperm surface, being particularly enriched in the postacrosomal region (Linder *et al.* 1995), and participates in the subsequent fusion of sperm and egg (Linder and Heinlein, 1997; Yuan *et al.* 1997). Orthologous genes from rat, monkey and human have also been cloned (Barker *et al.* 1994; Frayne *et al.* 1997; Adham *et al.* 1998).

Processing of the molecule to the 55 kDa form removes the N-terminal part of cyritestin, including the prodomain and the metalloprotease-like domain (Linder *et al.* 1995). The mature molecule consists of a disintegrin-like, EGF-like transmembrane and cytoplasmic domain. The disintegrin-like domain, as in other ADAM proteins, is thought to mediate cellular interaction as a ligand binding to an integrin in the membrane of the respective cellular counterpart (see Evans *et al.* 1995*a*; Huovila *et al.* 1996; Wolfsberg and White, 1996; Bigler *et al.* 1997), although the binding site is different from the classic arginine–glycine–aspartic acid motif (Linder and Heinlein, 1997; Yuan *et al.* 1997).

Localization of cyritestin in the acrosomal membrane has been determined by immunocytochemical methods in the absence and presence of detergents (Linder *et al.* 1995), but details of the integration of the protein into the acrosomal membrane and the time scale of processing are lacking. To address these questions, we have performed electron microscopic analyses using immunogold-labelling of antibodies directed against various parts of the cyritestin molecule. We show that the protein is delivered to the newly forming acrosomal vesicle *via* the Golgi apparatus. In addition, the lack of signal when using antibodies that recognize the metalloproteinase-like domain in germ cells later than stage 12

indicates that the processing of cyritestin takes place intratesticularly.

Materials and methods

Antibodies

Three different polyclonal antibodies from immunized rabbits were used, anti-225, anti-429 and anti-750. Either synthetic peptides or bacterially expressed protein fragments were used as antigens. Anti-225 was generated by injection of a synthetic peptide, S-E-Q-D-K-I-E-T-N-G-D-A-D-E-C, representing an evolutionarily less-conserved cyritestin amino acid sequence within the metalloprotease-like domain from residues 241 to 254 with an additional cysteine at the C terminus. For anti-750, the peptide used for injection was G-N-T-D-Q-N-F-M-T-V-P-G-S-F, which represents one of three repeats in the cytoplasmic domain corresponding to amino acids 746–759, 766–779 and 786–799 of cyritestin (see Heinlein *et al.* 1994; Linder *et al.* 1995; the accession number of the entire sequence is X64227). Anti-429 was generated by immunization with a recombinant metalloprotease-like domain expressed in bacteria. For this purpose, a 429 bp PvuII-fragment from cDNA clone TAZ83/13 was subcloned into the SmaI site of expression vector pQE32 (Qiagen). Recombinant His-tagged protein was purified by affinity chromatography on nickel–agarose, as described by Linder *et al.* (1995). Immunizations of rabbits were performed at Gamma S.A., Angleur, Belgium, and synthetic peptides were synthesized at Affiniti, Mamhead, UK.

Tissue preparation, fixation and embedding

Adult male C57BL/6J mice were killed by cervical dislocation. Testicles and epididymides were separately dissected and rinsed in Sorensen's phosphate buffer. Seminiferous tubules and epididymides were cut into smaller pieces and washed three times for 10 min in phosphate buffer before immersion for 3 h at room temperature (20–23 °C) in 4 % paraformaldehyde, 0.2 % glutaraldehyde and 1 mmol l^{-1} $CaCl_2$ in 0.1 mol l^{-1} phosphate buffer. After fixation, samples were again washed three times for 10 min in phosphate buffer, and then dehydrated in ethanol (15 min in 50 % and 70 % at 4 °C, 20 min each in 70 %, 80 %, 96 %, and two changes in 100 % at −20 °C). Dehydrated samples were then embedded at room temperature in LR Gold (LRG) resin as follows: LRG/ethanol (1:2), 30 min; LRG/ethanol (1:1), 30 min; LRG/ethanol (2:1), 30 min; LRG, 12 h; LRG, 24 h; LRG, 12 h. Samples were then placed in separate gelatine capsules, chilled to −20 °C and polymerized under ultraviolet light for 24 h at −20 °C.

Electron microscopy

Sections between 50 and 90 nm in thickness were cut with glass knives and mounted on coated nickel grids, where they were treated with antibodies before transmission electron microscopy using a Zeiss EMi 9. For antibody labelling, the mounted sections were first blocked for 30 min with 0.25 % (w/v) bovine serum albumin (BSA-C), 0.1 % Tween-20 and 0.1 mol l^{-1} glycine in phosphate-buffered saline (PBS), and then incubated with specific antibody diluted in PBS. Samples were then washed three times for 10 min in 0.25 % BSA-C and 0.1 % Tween-20 in PBS, and twice for 10 min in PBS.

Immunocytochemistry

Mouse spermatozoa were obtained from dissected vas deferens, air-dried on glass slides, fixed in 4 % formaldehyde, washed twice in PBS, and treated with 1 % Triton X-100 in PBS for 5 min. After two rinses in PBS, anti-750 serum (1:50 in PBS) was applied for 2 h at room temperature in a wet chamber. After repeated washing steps in PBS, Texas-Red-conjugated goat anti-rabbit antibodies were added for 1 h. After thorough rinsing in PBS, the slides were air-dried and then mounted in Citifluor.

Results

Ultrathin sections of fixed mouse seminiferous tubules were analyzed by electron microscopy to localize cyritestin by immunogold staining. The two goals of this study were (i) to determine the precise intracellular localization of the protein in germ cells, and (ii) to investigate the timing of proprotein processing, i.e. the removal of the N-terminal part of the molecule containing the prodomain and the metalloprotease-like domain. The latter aspect was investigated by using different antibodies, one recognizing the metalloprotease-like domain and another specific for the cytoplasmic domain. Positive immunostaining with both antibodies would indicate the presence of the complete cyritestin molecule, while the absence of a metalloprotease-specific signal would suggest removal of the N-terminal domain, as detected by western blotting experiments (Linder *et al.* 1995). Table 1 summarizes the findings with regard to immunogold signal generation for the different antibodies. Fig. 1 illustrates the acrosomal localization of the molecule in mature, post-epididymal spermatozoa as demonstrated by immunocytochemistry and

Table 1. *Summary of positive immunogold reactions*

Stage	Pro- anti-225	Met-prot anti-429	Cytopl anti-750
2	+	+	+
3	+	+	+
5	+	+	+
7	+	+	+
9	+	+	+
12	+	+	+
15	–	–	+
>16	–	–	+

Antibodies anti-225, anti-429 and anti-750 recognize the prodomain (Pro), metalloprotease-like (Met-prot) domain and cytoplasmic (Cytopl) domain of cyritestin, respectively.

Spermatogenic stages were defined as described by Russell *et al.* (1990).

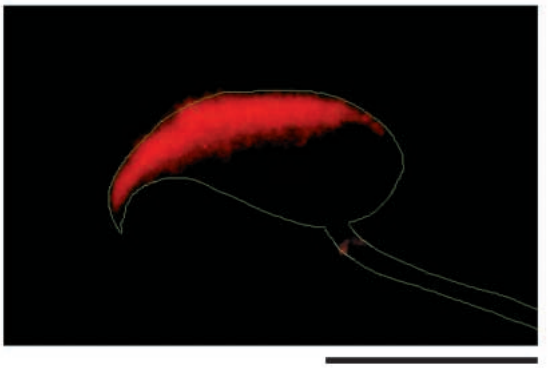

Fig. 1. Cyritestin localization in the acrosome of mature epididymal spermatozoa. Spermatozoa were collected, detergent-treated (Linder *et al.* 1995) and incubated with anti-750 antibody directed against the cytoplasmic moiety of cyritestin. Bound antibody was detected with a Texas-Red-conjugated secondary antibody and observed under a fluorescence microscope. The shape of the sperm head (outlined in green) was added to the digitized image. Scale bar, 10 µm.

the photomicrographs in Figs 2–5 provide examples of the signals obtained.

A comparison of Fig. 2A with Fig. 2B provides evidence that proteolytic processing of the molecule takes place intratesticularly. The anti-429 antibody specific for the metalloprotease-like domain failed to label the mature (stage 15) spermatozoa (Fig. 2B), although the presence of cyritestin could be demonstrated using anti-750, the antibody to the cytoplasmic domain. In contrast, anti-429 yielded positive signals in the acrosomal vesicle of stage 7 spermatids (Fig. 3). An antibody directed against the cytoplasmic domain of the transmembrane protein, however, generated immunogold signals in stage 15 spermatids as well as in elongating spermatids and elongated spermatozoa (see Fig. 2). Corresponding results were obtained with antibody anti-225 directed against a synthetic peptide from the metalloprotease-like domain. All three antibodies generated signals through stages 2–12, but only anti-750 led to detectable gold particles in acrosomes of elongated spermatids and mature spermatozoa later than stage 12 (see Table 1).

As expected from previous immunofluorescence (see Fig. 1) and biochemical (Linder *et al.* 1995) studies, the majority of gold particle deposition was observed in the acrosomal region of germ cells. In elongated spermatids and mature spermatozoa, the gold label was specifically located over the acrosomal membranes (Figs 4, 5), while in less mature germ cells, where the acrosome was still forming, additional signals could be detected in vesicle-like structures, probably Golgi vesicles transporting material to the acrosome (see Fig. 6). These vesicular signals were detected mainly during stages 2–3, i.e. during the formation of the acrosomal vesicle itself, and occurred at much lower frequency during stages 4–7.

A difference in gold particle distribution was observed for anti-429 and anti-750 antibodies. While anti-750, recognizing the cytoplasmic domain of cyritestin, bound to its antigen along the acrosomal membranes (Figs 4, 5), anti-429 antigen,

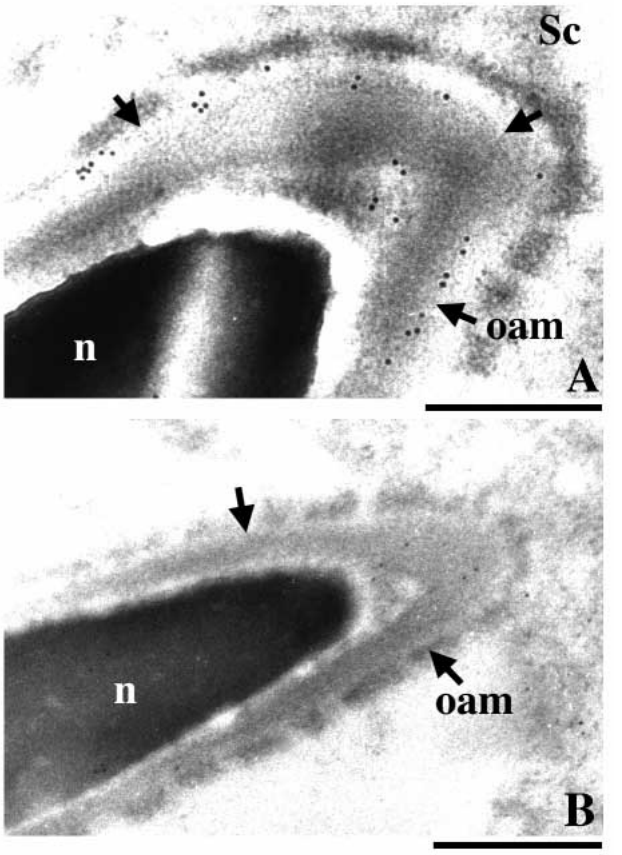

Fig. 2. Localization of cyritestin in the acrosomes of intratesticular mature, elongated spermatozoa. Scale bars, 1 µm. (A) Immunogold detection in a testicular spermatozoon (stage 15) using anti-750, which recognizes the cytoplasmic domain, as the primary antibody (see Fig. 1). n, nucleus; oam, outer acrosomal membrane; Sc, Sertoli cell. (B) Lack of immunogold signal after incubation of a testicular spermatozoon (stage 15) with anti-429 antibody directed against the metalloprotease-like domain of cyritestin.

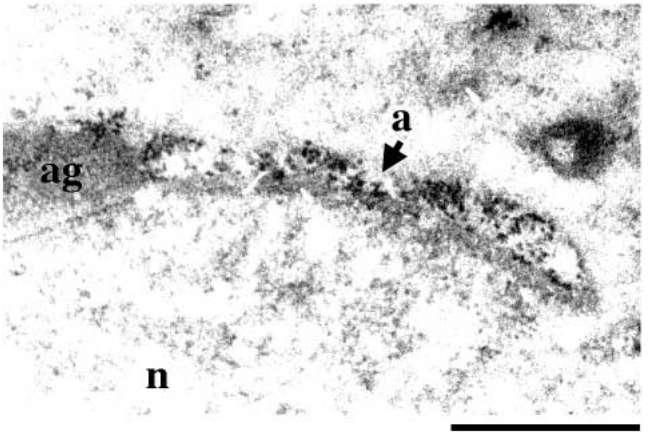

Fig. 3. Immunogold detection of the metalloprotease-like domain in a stage 6–7 spermatid, using anti-429 as a primary antibody. ag, acrosomal granule; a, acrosome; n, nucleus. Scale bar, 1 µm.

the metalloprotease-like domain, was more evenly distributed over the acrosomal lumen (see Fig. 3).

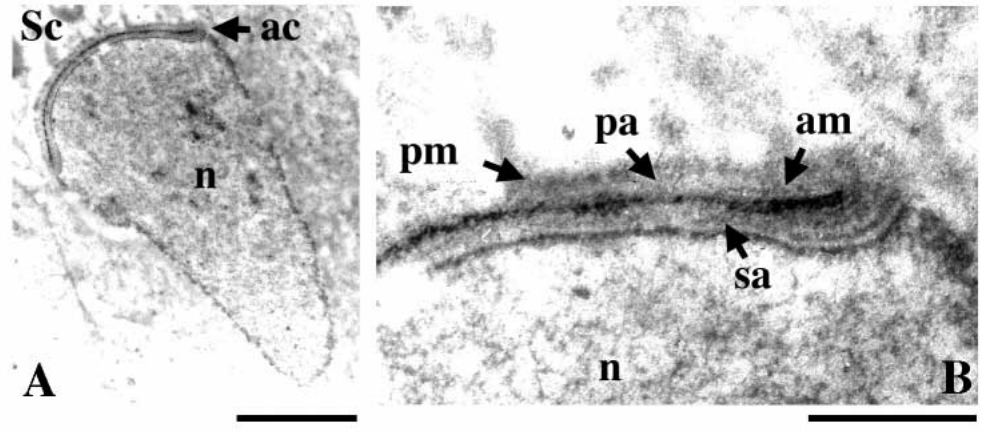

Fig. 4. Localization of cyritestin in a stage 10 elongated spermatid using anti-750 as the primary antibody. (A) Entire sperm head cross-section. Sc, Sertoli cell; ac, acrosome; n, nucleus. Scale bar, 2 μm. (B) Enlarged portion of A. pm, plasma membrane; pa, periacrosomal layer; am, acrosomal membranes; sa, subacrosomal layer; n, nucleus. Scale bar, 0.5 μm.

Discussion

In the present study, we used an electron microscopic approach to determine the intratesticular localization of cyritestin, a transmembrane protein of the mouse acrosome involved in gamete interaction (Heinlein *et al.* 1994; Linder *et al.* 1995; Linder and Heinlein, 1997; Yuan *et al.* 1997). Cyritestin belongs to the ADAM family of proteins, members of which are characterized by the presence of metalloprotease-like, disintegrin-like, cysteine-rich and EGF-like domains. As shown by several studies, ADAM proteins are generally processed post-translationally by proteolytic removal of the N-terminal portion of the polypeptide, including the metalloprotease-like domain, leading to membrane-bound molecules consisting of disintegrin-like, cysteine-rich and EGF-like extracellular domains. For two members of the protein family, fertilin β and cyritestin, the disintegrin domain has been shown to be a putative ligand for integrin receptors on the egg membrane, indicating that the processing step is a prerequisite for the generation of fertilization-competent spermatozoa.

The biochemical reaction leading to the processing has yet to be determined. It is possible that removal of the N-terminal

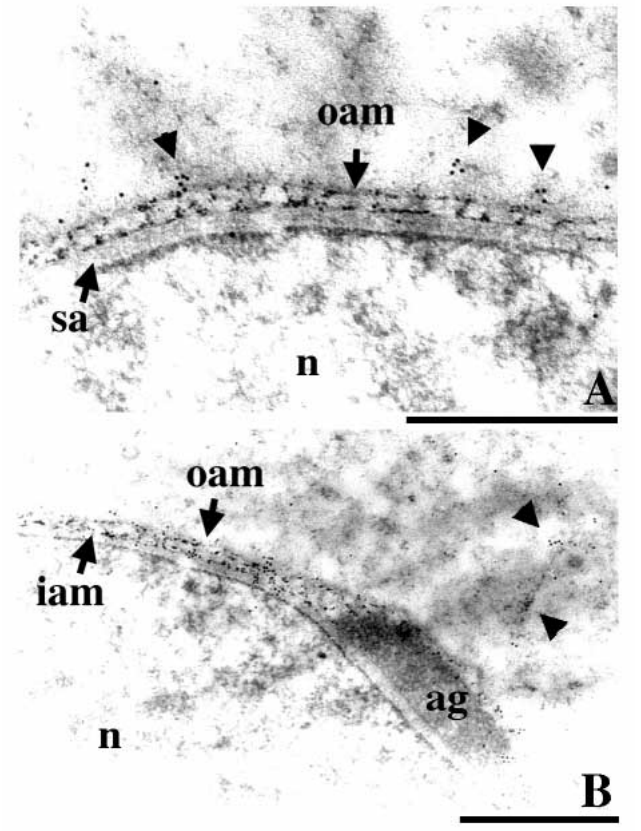

Fig. 5. Cyritestin localization in the acrosomal membrane of a stage 7 spermatid using anti-750 as the primary antibody. Scale bars, 1 μm. (A) Gold particles are deposited along the acrosomal membranes. oam, outer acrosomal membrane; sa, subacrosomal layer; n, nucleus. Arrowheads identify gold particle clusters representing vesicles delivering cyritestin. (B) Positive immunogold reaction of the acrosomal membranes, but no signals in the acrosomal granule. oam, outer acrosomal membrane; iam, inner acrosomal membrane; ag, acrosomal granule; n, nucleus.

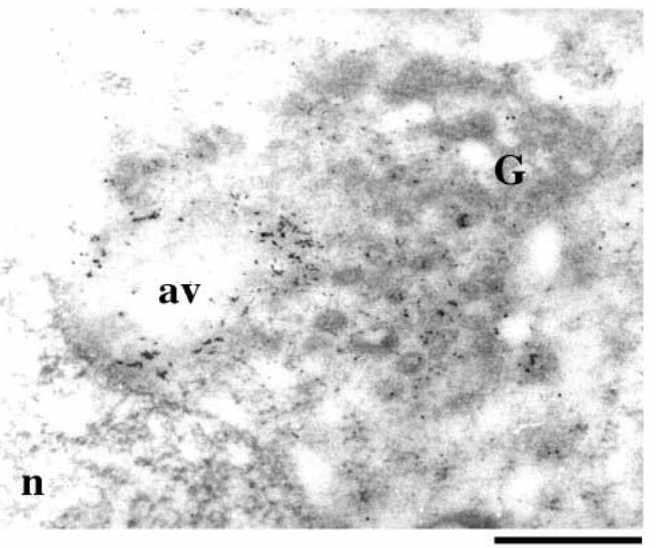

Fig. 6. Cyritestin molecules are delivered to the acrosomal vesicle of a stage 2–3 spermatid before the acrosomal membranes have formed. Anti-750 was used as the primary antibody. G, Golgi apparatus; av, acrosomal vesicle; n, nucleus. Scale bar, 1 μm.

domain is achieved by the action of specific proteases or, as an attractive alternative, the molecule may be subject to autoproteolysis performed by the metalloprotease-like domain. In the cases of fertilin β and cyritestin, however, the histidine residues that are normally involved in binding the zinc atom and are characteristic of other metalloproteases of this particular type are lacking. This suggests that these two molecules at least are not capable of performing autoproteolytic processing. In guinea pig and bovine sperm, fertilin β is known to exist as a dimer with fertilin α, which possesses these histidine residues and thus might function as a processor for fertilin β. In the case of cyritestin, no dimeric partner has yet been detected, but the possibility remains that such a partner may exist.

The results presented in this study provide evidence for the timing of this processing event. Differential immunogold staining with antibodies recognizing the metalloprotease-like domain and the cytoplasmic domain, respectively, resulted in a complete loss of signal from the metalloprotease-like domain in germ cells later than stage 12. Germ cell stages are usually evaluated by several parameters, including the dimensions of the forming acrosome (see Russell *et al.* 1990). This peculiar structure derives from the Golgi apparatus, from where an initial acrosomal vesicle is launched which adheres to the nuclear membrane and starts to expand into a characteristic acrosomal cap. As shown in Figs 4 and 5, cyritestin is delivered to the acrosomal structure during its initial formation. Gold particles were detected in Golgi-derived vesicles and along the acrosomal vesicle. This suggests that the deposition of cyritestin and comparable acrosomal proteins relies critically on the exact time of translation, since they would otherwise not be transported correctly. An indication for this comes from studies which investigated the timing of *Cyrn1* gene transcription and translation of the mRNA. While *Cyrn1* transcripts occur in the germ cells as early as 14 days after birth, the mRNA is translated 3–4 days later, around postnatal day 17–18 (Linder *et al.* 1995). Thus, translational control is obviously taking place to ensure that the protein is synthesized at an appropriate time to be transported to the prospective acrosome *in statu nascendi*. Although we were unable to detect gold-decorated Golgi-like vesicles after the acrosomal cap had extended over more than 90°, corresponding to stages 6 and later, we cannot completely exclude the possibility that more cyritestin protein might be delivered to the mature acrosomal structure at a very low rate, but we consider this to be unlikely.

These data accord with observations made in the rat by Clermont and Tang (1985) that also suggested that the acrosome no longer accepts proteins from the Golgi after stage 7. At that stage, the Golgi apparatus starts to migrate to the caudal region of the condensing spermatid (see Peterson *et al.* 1992). It is not clear which events cause the shut-down of Golgi–acrosome communication. It could either be interrupted by changes in the Golgi and the endoplasmic reticulum, e.g. by differential processing or decreased translation rates, or by changes in the acrosomal membrane itself that prevent further vesicle fusion. Our results show that there is a decrease in the amount of proteins, such as cyritestin, transported, suggesting that regulation at the translational or protein-processing levels might be primarily responsible for the cessation of protein integration into the acrosome rather than some modification of the acrosomal membrane.

The processing of pro-cyritestin to its mature form involves the removal of the N-terminal part, including the metalloprotease-like domain (Linder *et al.* 1995), leaving behind the functional molecule consisting of a disintegrin-like, an EGF-like, a transmembrane and a cytoplasmic domain. The results with anti-429 antibody, shown in Fig. 3, suggest that this processing takes place in the acrosome soon after integration of cyritestin into the membrane, because the gold particles appear to be distributed over the acrosomal lumen and probably represent free, clipped-off pro-/metalloprotease domains. As mentioned above, the metalloprotease-like domain of cyritestin lacks the histidine residues necessary for a putative protease activity, suggesting that the clipped-off domains do not perform any special enzymatic tasks in the acrosomal lumen.

In a study published by Yuan *et al.* (1997), cyritestin was immunocytochemically localized on the equatorial domain of acrosome-intact as well as acrosome-reacted sperm plasma membranes, which contrasts with the results obtained by Linder *et al.* (1995), who showed that plasma membrane immunoreactivity was dependent on a successful acrosome reaction. The results presented in this paper show that the transmembrane protein cyritestin is delivered to the acrosome by vesicular transport from the Golgi, becoming a component of the acrosomal membrane(s) and, as a consequence, it would appear that exposure of the N-terminal 'extracellular' part of the protein on the outside of the sperm plasma membrane would only be possible after fusion of the acrosomal and plasma membranes, i.e. after the acrosome reaction. These findings therefore support the results of Linder *et al.* (1995), but still do not explain in detail the reasons for the discrepancy between the studies mentioned above.

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