ORIGINAL ARTICLE

Occurrence of calreticulin during the exchange of nucleohistones into protamine-type proteins in *Chara vulgaris* spermiogenesis

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Abstract During spermiogenesis of an alga *Chara vulgaris*, which resembles that of animals, nucleohistones are replaced by protamine-type proteins. This exchange takes place in a spermatid nucleus during the key V spermiogenesis stage, in which rough endoplasmic reticulum is the site of protamine-type protein synthesis and is also the pathway guiding the proteins to their destination, nucleus. In the present work, it was shown that a chaperon protein, calreticulin (CRT), abundantly present at this significant V stage of spermiogenesis in a few cellular compartments, i.e., a nucleus, lumen of cisternae, and vesicles of significantly swollen ER as well as outside these structures, e.g., in Golgi apparatus, could have taken part in the process of exchange of nuclear proteins. Colocalization of two proteins, protamine-type proteins, crucial for reproduction, and CRT, was especially visible in a nucleus, mainly on its peripheries where condensed chromatin was present. Localization of protamine-type proteins and CRT in nucleus is in agreement with our previous results showing that protamine-type proteins were twofold more labelled in the peripheral area in comparison to the nucleus center occupied by noncondensed chromatin. The role of CRT in the reproduction of both plants and animals is also discussed.

Keywords Calreticulin · ER cisternae and vesicles · Immunogold technique · Nucleohistones · Protamine-type proteins · Spermiogenesis

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Introduction

Spermiogenesis is one of the key processes in animal and some plant organisms. This period is characterized by chromatin remodelling; nucleohistone structure, characteristic of somatic cells, is replaced by fibrillar chromatin resulting in gene silencing in mature spermatozoids (Ward 1994). This process leads to extreme chromatin condensation (Pogany et al. 1981; Ward and Coffey 1991; Daban 2000; Ausio et al. 2007) and results from the exchange of histones into protamines or other strongly basic proteins (Steger 1999; Dadoune 2003; Balhorn 2007; Worawittayawong et al. 2008; Hecht et al. 2009; Kurtz et al. 2009; Miller et al. 2010). It is a common process mostly in the case of animals from non-vertebrates, e.g., in *Drosophila melanogaster* (Rathke et al. 2007), and fish (Oliva and Dixon 1991; Kurtz et al. 2009) to mammals, including humans (Wouters-Tyrou et al. 1998; Ramos et al. 2008). In the case of plants, it takes place only in the organisms producing free-moving spermatozoids in water, e.g., in *Marchantia polymorpha* (Reynolds and Wolfe 1978) and in *Marsilea vestitia* (Reynolds and Wolfe 1984). The exchange of somatic, nucleus-specific histones with protamine-type proteins was also described in higher algae, in *Chara corralina* (Reynolds and Wolfe 1984), *Chara vulgaris* (Kwiatkowska and Popłońska 2002; Popłońska et al. 2007), as well as in *Chara tomentosa* (Kwiatkowska et al. 2002; Kwiatkowska and Popłońska 2003; Popłońska et al. 2007).

   Light and electron microscopy studies of the two latter *Chara* species were allowed to differentiate ten spermiogenesis stages (I–X) (Kwiatkowska and Popłońska 2002; Kwiatkowska et al. 2002). Both cytochemical (Popłońska 2002) and immunocytochemical (Popłońska et al. 2007) studies of the exchange of histone proteins into protamine-type proteins in both *Chara* species showed that at early stages (I–IV) only histones were present while at final stages

(IX–X) only protamine-type proteins were observed in the nuclei of differentiating spermatozoids. Moreover, the capillary electrophoresis with mature *C. tomentosa* spermatozoid extract confirmed lack of histones which were replaced by three basic protein fractions (with molecular weights 9.1, 9.6, and 11.2 kDa), with motility similar to that of salmon protamines. These analyses revealed small amounts of protamine-type proteins already in mid-spermiogenesis, and during the late spermiogenesis, they almost reached the maximum level. However, during *C. tomentosa* spermiogenesis, no transient proteins were observed (Kwiatkowska et al. 2002), as contrasted for example with mammals (McLay and Clarke 2003). Using Western blot method showed additional protamine-type proteins with molecular weights 3–6 kDa (Popłońska et al. 2009).

The V stage of *Chara* spermiogenesis is the starting point of the exchange of nucleohistones into nucleoprotamines, which is pivotal for proper chromatin condensation, leading to extremely condensed chromatin in mature spermatozoids. Ultrastructural studies of both *Chara* species revealed that, in the V stage, the extensive system of ER vesicles and cisternae connected with a nuclear envelope was present. Both the vesicles and the swollen cisternae are filled with dark, granular content, and the intermembrane area of a nuclear envelope also contains similar homogenous substance (Kwiatkowska 1996; Kwiatkowska and Popłońska 2002, 2003; Kwiatkowska et al. 2002). The cytochemical and immunocytochemical ER studies revealed clear staining and strong antigenic signals against protamine-type proteins which were observed in the form of parallel strands in the cytoplasm near spermatid nuclei (Popłońska et al. 2007), which confirmed the earlier hypothesis that protamine-type protein synthesis may occur in ER area (Kwiatkowska 1996; Kwiatkowska and Popłońska 2002; 2003; Kwiatkowska et al. 2002). The immunogold method revealed gold labelling of ER vesicles and cisternae at the V stage of spermiogenesis. Therefore, both these subdomains are the site of protamine-type protein synthesis which are subsequently transported directly to the nuclear envelope, and via endocytosis by invagination of an internal membrane of the nuclear envelope, they reach their destination, that is, a nucleus (Popłońska et al. 2009).

ER plays a crucial role in many cell processes, including storage and release of Ca^{2+}, lipid and protein synthesis, as well as in proper folding, and post-translational modifications of the latter ones (Baumann and Walz 2001; Hebert and Molinari 2007; Michalak et al. 2009). In the ER lumen, there are perfect conditions for folding and maturation of proteins. There are great amounts of dissolved enzymatic proteins and molecular chaperons; all of them are referred to as reticuloplasmins. Among these reticuloplasmins residing in ER, there is calreticulin (CRT) which is a chaperon present both in animal (Persson et al. 2002; Park et al. 2001) and plant cells (Šamaj et al. 2008; Lenartowska et al. 2009; Jia et al. 2009). The plant CRT was extracted from spinach leaves (Menegazzi et al.

1993), barley (Chen et al. 1994), tobacco (Denecke et al. 1995), maize (Dresselhaus et al. 1996; Kwiatkowski et al. 1995), Chinese cabbage (Lim et al. 1996), *Arabidopsis* (Nelson et al. 1997), rice (Li and Komatsu 2000), and wheat (Jia et al. 2008). These CRTs showed high homology to their counterparts in mammals. There are some indications that CRT may also be involved in reproductive events in plants, e.g., in fertilization (Dresselhaus et al. 1996; Nelson et al. 1997; Williams et al. 1997) and embryo development (Chen et al. 1994; Borisjuk et al. 1998).

The present research aimed:

1. To determine the CRT levels at three selected stages (IV, the stage before the exchange of nuclear proteins; V, at the beginning of exchange, and VIII, at the end of exchange) of *C. vulgaris* spermiogenesis.
2. To analyze in which spermatid domains CRT is cumulated.
3. To check whether CRT colocalizes with protamine-type proteins at the crucial V stage of spermiogenesis.

Materials and methods

Apical parts of *C. vulgaris* L. *thallus* were obtained from plants grown in an artificial pond located in Rogów Arboretum. Antheridia were taken from III–V pleuridia node counting from the top. Before the onset of the experiment, the plants were cultivated for a few days in tanks containing water from the natural environment at the photoperiod similar to natural, i.e.,

L : D = 14 : 10.

Western blot technique

C. vulgaris antheridia were isolated, selected (only spermatids from the V stage of spermiogenesis were chosen), and kept in −20°C. Reassembled antheridia were gently crashed in a cold glass mortar with 10 mM Tris–HCl saline buffer (pH 8.0) supplemented with 10 mM NaCl, 5 mM $MgCl_2$, 0.25 M sucrose, 2 mM DTT, and cocktail of protease inhibitor (Sigma). To separate antheridial filament cells from shield cells, the crashed antheridia were repeatedly washed with the buffer using Pasteur pipette. During the last washing, the antheridial filament cells were collected by centrifugation at 1,000×g for 15 min (4°C). Proteins were extracted from antheridial filament cells with the P-PER Plant Protein Extraction Kit (Pierce) according to the protocol and then were fractionated on 4–12% Bis Tris/MES PAGE-SDS gels (Novex) and blotted onto PVDF Membrane 0.2 μm pore size (Invitrogen). Anti-CRT (anti-calregulin (N-19): goat polyclonal antibody sc-6468 raised against calregulin of human origin, Santa Cruz Biotechnology) diluted 1:200 was detected

using chromogenic Western blot immunodetection kit (Invitrogen). Instead of the primary antibody, purified antibodies from non-immune goat serum were used as a control at the same concentration as the specific antibody.

Single labelled immunogold technique

C. vulgaris plants were fixed with 0.5% glutaraldehyde and 2.5% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.3) supplemented with 0.007 M $CaCl_2$ for 3 h at RT. The antheridia were isolated and gently squashed in a drop of heated 2% agar in cacodylate buffer. After dehydration in the ethanol series, the material was embedded in Epon 812 and Spurr mixture medium (Polysciences). Embedded antheridia of three spermiogenesis stages: early (IV), middle (V), and late (VIII) were selected. Freshly cut ultrathin sections were mounted on nickel Formvar-coated grids for EM investigations. Air-dried grids with sections were blocked with 0.5% bovine serum albumin and 0.05% Tween 20 in phosphate buffered saline (PBS) (0.01 M, pH 7.4, Sigma) for 10 min and then dried with tissue-paper. The sections on grids were incubated overnight at 20°C with the primary antibody (anti-calregulin, Santa Cruz Biotechnology) diluted in an antibody diluent (pH 8.0, DAKO) at 1:25 dilution. Then the grids were washed five times for 5 min each with the same PBS and incubated with the secondary antibody (rabbit anti-goat IgG conjugated with 20 nm gold [no. EM. RAG20, Polysciences]) diluted 1:70 in the antibody diluent for 1.5 h in the same temperature and rinsed again in PBS and distilled water (ten times for 5 min each). After this, sections were double-stained with uranyl acetate and lead citrate according to Reynolds (1963). The sections were also examined and photographed in a JEOL JEM-1010 transmission electron microscope.

Double labelled immunogold technique

C. vulgaris plants were fixed as above. To isolate antheridia and to prepare the material for embedding in mixture medium (Polysciences), the similar techniques to those above were used. The antheridia in the crucial middle stage of spermiogenesis (V) were selected. Further steps were similar to the single labelled immunogold technique. The sections on grids were incubated overnight at 20°C with the antibody (anti-calregulin, Santa Cruz Biotechnology) diluted in an antibody diluent (pH 8.0, DAKO) at 1:25 dilution and were incubated with the antibody (rabbit anti-goat IgG conjugated with 10 nm gold [no. EM. RAG20, Polysciences]) diluted 1:70 in the antibody diluent for 1.5 h in the same temperature. Next, the above procedure was repeated for rabbit polyclonal antibody to the protamine-type proteins isolated from antheridia of *C. tomentosa* (technique of isolation; see Popłońska et al. 2007) diluted in an antibody diluent (pH 8.0, DAKO) at a 1:250 dilution and incubated with the final antibody (anti-rabbit IgG

conjugated with 20 nm gold [no. EM.GAR20, Polysciences]) diluted 1:70 in the antibody diluent for 1.5 h in the same temperature.

Analysis of spermatid immunolabelling

Micrographs of 30 spermatids from the IV, V, and VIII stage were analyzed. Statistical results were estimated by Student *t* test with the use of Microsoft Excel 2000. Differences in number of grains in the studied compartments between IV and V stage as well as between V and VIII stage of spermiogenesis are statistically significant taking $p \leq 0.05$ as the significance level. To show colocalization of protamine-type proteins with CRT, micrographs of 35 spermatids from the V stage were analyzed. The percentage of gold grains reflecting the colocalization of protamine-type proteins with CRT in each cellular compartment was calculated taking as 100% for grains the whole cell, as well as percentage of gold grains reflecting the colocalization of protamine-type proteins with CRT in individual cellular domain was calculated taking as 100% for grains in the given cellular domain. Statistical results were also analyzed with the use of Microsoft Excel 2000, and means±SE were estimated.

Results

Western blot analysis

Western blot was performed with the use of an antibody raised against human CRT with molecular weight about 55 kDa. In the extract from *C. vulgaris* antheridia from the V stage of spermiogenesis, two bands pointing proteins with molecular weights of about 55 and 62 kDa were revealed (Fig. 1a, line 3). The comparison with the control serum (Fig. 1a, line 4) indicates that faint band with molecular mass about 70 kDa did not originate from CRT polypeptide. This suggests that CRT exists in two isoforms in the studied material. Moreover, this result indicates that this antibody is specific and could be applied for the immunogold technique in our plant material.

Immunogold analyses of ultrathin sections

The same antibody, previously checked with Western blot technique, was used in immunogold analyses. At the early spermiogenesis stage (IV), both the cytoplasm and nuclei were weakly immunolabelled (Fig. 1b and Table 1). Spermatids in which the antibody to CRT was omitted were not labelled (negative control; Fig. 1c).

At the V stage—crucial for spermatozoid maturation—gold grains were mainly observed in the nuclei, in the extensive system of ER cisternae and vesicles (Figs. 1d, 2a, b, and 3a and
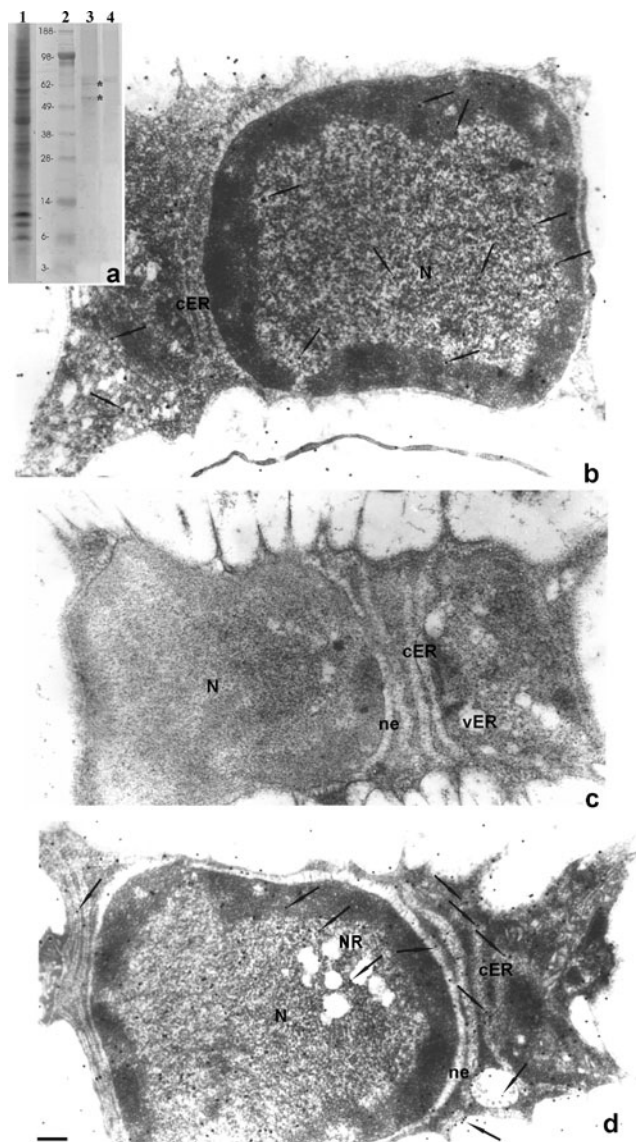
Fig. 1 Immunodetection of CRT during spermiogenesis in *C. vulgaris*. **a** Western blot analysis; *line 1*, SDS-PAGE electrophoretic separation of the antheridial filament cells extract; *line 2*, molecular mass standards and their weights in kilodaltons; *line 3*, Western blotting of the antheridial filament cells extract probed with the anti-human CRT antibody; *line 4*, CRT pre-immune serum; *Proteins revealed **b** ultrastructure of spermatid at IV stage of spermiogenesis in longitudinal section (immunogold technique). **c** Spermatid at V stage of spermiogenesis (negative control to immunogold technique). **d** Ultrastructure of spermatid at V stage of spermiogenesis in longitudinal section (immunogold technique). *Arrows* show localization of gold grains in particular spermatid compartment. *cER*, endoplasmic reticulum cisternae; *vER*, endoplasmic reticulum vesicles; *N*, nucleus; *ne*, nuclear envelope; *NR*, nuclear reticulum; *Bar*=250 nm

Table 1 Number of gold grains after the use of antibody to CRT in particular *C. vulgaris* spermatid compartments at stages IV, V, and VIII of spermiogenesis

	Stage IV	Stage V	Stage VIII
Compartment		Number of grains	
Endoplasmic reticulum			
Vesicles	12±1.45	46±1.02	−
Cisternae	11±0.85	21±0.72	−
Total	23±1.04	67±1.35	8±0.8
Whole nucleus	42±0.45	127±1.4	4±1.6
Nuclear envelope	5±0.32	14±0.38	−
Chromatin			
Condensed	20±1,22	61±1.03	−
Noncondensed	17±0.9	52±0.95	−
Total	37±0.78	113±1.42	−
Cytoplasm+other organelles	12±1.3[a]	11±0.43[a]	3±0.9

Values represent the mean±SE; differences in number of grains in the studied compartments between IV and V stage as well as between V and VIII stage of spermiogenesis are statistically significant taking $p \leq 0.05$ as the significance level
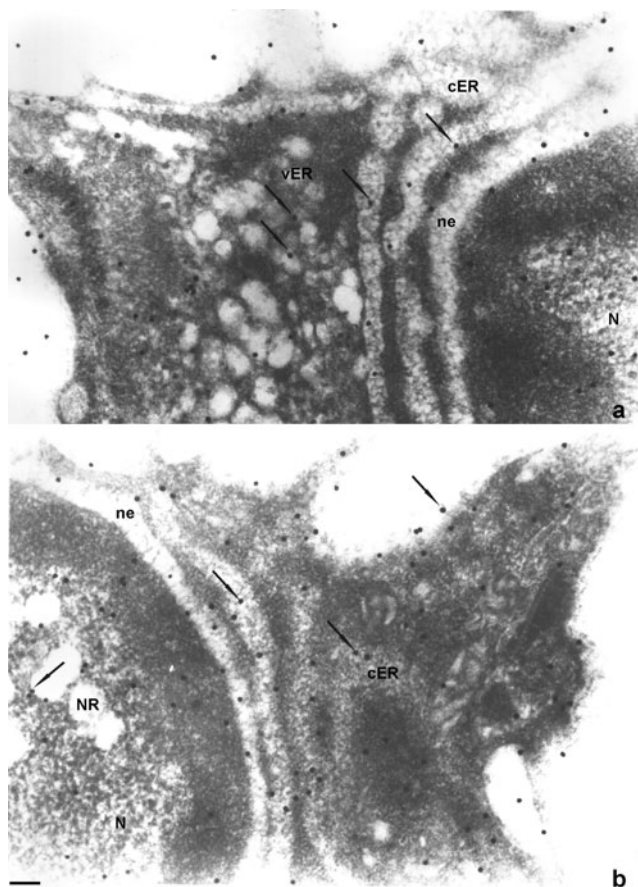
[a] These differences are not statistically significant

noncondensed chromatin was located (Fig. 1d) as well as in the nuclear envelope connected with swollen rough endoplasmic reticulum (Fig. 2a, b) filled with protamine-type proteins (Popłońska et al. 2009). In noncondensed chromatin, there were aggregates of vesicles called nuclear reticulum resulting from invagination of an inner membrane of the nuclear envelope. These structures contained gold grains which were located near their peripheral part (Figs. 1d and 2b). At late spermiogenesis stage (VIII), few gold grains were identified only at plasmalemma and in poorly developed ER cisternae of significantly reduced cytoplasm (Fig. 3b, grey arrows). Within a crescent nucleus fibrillar chromatin (fibrils parallel to the nucleus long axis), no gold grains were observed, while between fibrils, where electron density was lower, few immunosignals were present (Fig. 3b, white arrows).

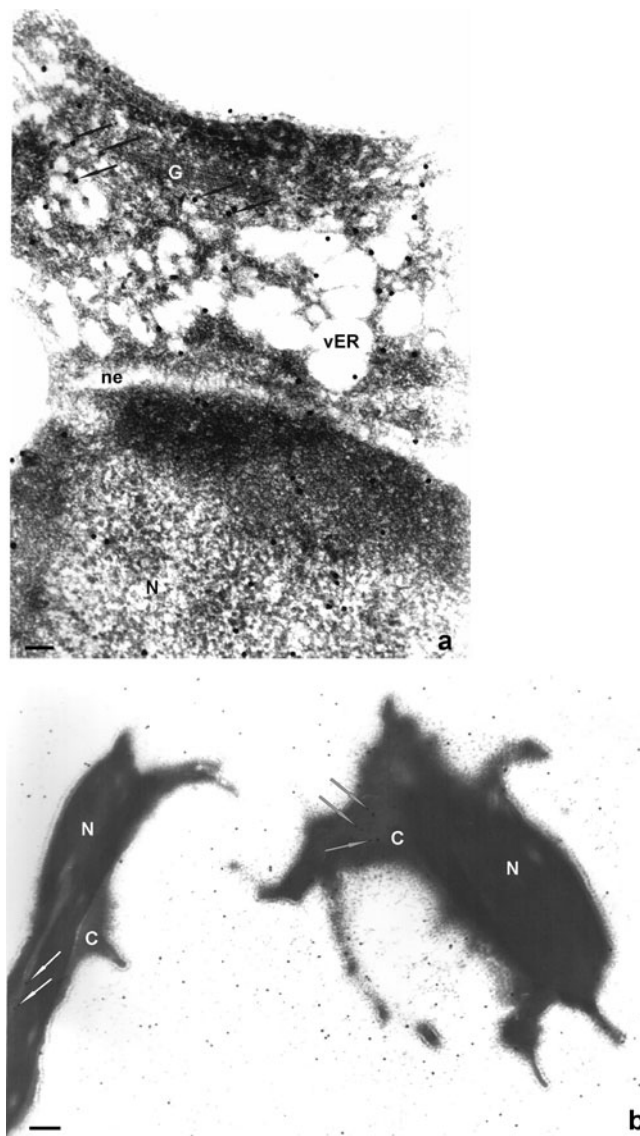## Colocalization of protamine-type proteins and CRT

For double immunogold labelling, anti-CRT antibody was used as the first antibody, and antibody against protamine-type proteins isolated from *C. tomentosa* antheridia followed. Spermatids in which the antibodies to the studied proteins were omitted were not labelled as before (negative control, not shown).

At the V stage, where the CRT level was the highest, gold grains from both antibodies were mostly visible in nuclei, extensive system of ER cisternae, and vesicles (Fig. 4a, b) and in Golgi apparatus (Fig. 4b). Double-

Table 1) and at the plasmalemma (Figs. 1d and 2b), in the Golgi apparatus (Fig. 3a), as well as in the cell walls of adjacent spermatids (not shown). Labelling from a nucleus was observed in its peripheries adjacent to the nuclear envelope where condensed chromatin was present and at its central part where

Fig. 2 Ultrastructure of spermatid at V stage of spermiogenesis in longitudinal section (immunogold technique for anti-CRT). **a-b** *Arrows* show localization of gold grains in particular spermatid compartment. *cER*, endoplasmic reticulum cisternae; *vER*, endoplasmic reticulum vesicles; *N*, nucleus; *ne*, nuclear envelope; *NR*, nuclear reticulum. *Bar*=100 nm

Fig. 3 Immunogold detection of CRT at stages V (**a**) and VIII (**b**) of *C. vulgaris* spermiogenesis. *C*, cytoplasm; *G*, Golgi apparatus; *N*, nucleus; *ne*, nuclear envelope; *vER*, endoplasmic reticulum vesicles; *black arrows* indicate immunolabelling of Golgi apparatus; *grey arrows*, only a few immunosignals in cytoplasm, and *white arrows*, sparse gold grains between fibrils of chromatin within a crescent nucleus. *Bar*= 100 nm (**a**) and *bar*=250 nm (**b**)

staining originating from the nucleus was most pronounced within condensed chromatin at its periphery just under the nuclear envelope and accounted for 27% of all protamine-type protein labelling in all cellular compartments (i.e., protamine-type proteins with CRT and protamine-type proteins alone comprise together 100% of grains) (Table 2) and 79% of all protamine-type proteins labelling in this part of a nucleus (grains from an individual cellular domains comprise 100%) (Fig. 5). In noncondensed chromatin in the nuclear center and in the nuclear envelope, both types of grains were less numerous and respectively accounted for 13% and 4% of all protamine-type proteins labelling in all cellular compartments (Table 2) and for 68% and 54% of all protamine-type proteins labelling in this specific region (Fig. 5). In ER, stronger colocalization was observed in vesicles than in cisternae—14% and 5% of overall protamine-type proteins, respectively (Table 2), and 50% and 63% of protamine-type proteins labelling in ER vesicles and cisternae (Fig. 5).

Discussion

In plants, there can be two or more CRT isoforms (Chen et al. 1994; Kwiatkowski et al. 1995; Nelson et al. 1997). Phylogenetic studies and expression analysis carried by Persson et al. (2003) revealed that *Arabidopsis* CRT1 and CRT2 form one group, whereas CRT3 constitutes another. These data strongly indicate that higher plants contain at least two distinct groups of CRTs: CRT1/CRT2 group and CRT3 group. Two CRT groups were also identified in the wheat genome (Jia et al. 2008). Western blot analyses of the
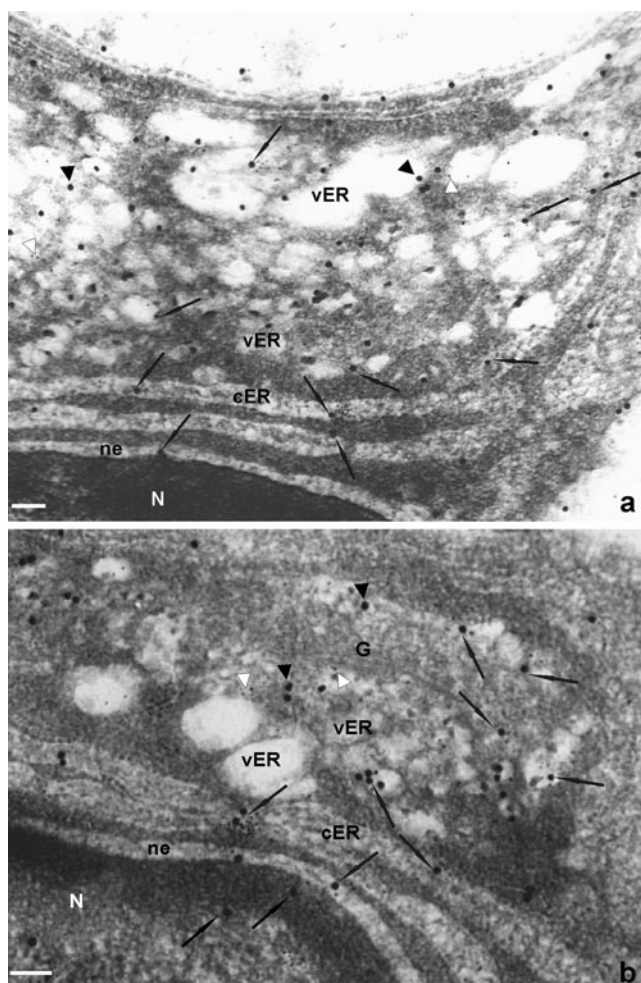
Fig. 4 Colocalization of protamine-type proteins (20 nm gold, *black arrowheads*) and CRT (10 nm gold, *white arrowheads*) at V stage of *C. vulgaris* spermiogenesis. **a-b** *Arrows* show common localization immunosignals for protamine-type proteins and calreticulin in particular spermatid compartment. *cER*, endoplasmic reticulum cisternae; *vER*, endoplasmic reticulum vesicles; *G*, Golgi apparatus; *N*, nucleus; *ne*, nuclear envelope; *Bars*=100 nm

Table 2 The percentage of protamine-type protein colocalization with CRT during the V stage of *C. vulgaris* spermiogenesis

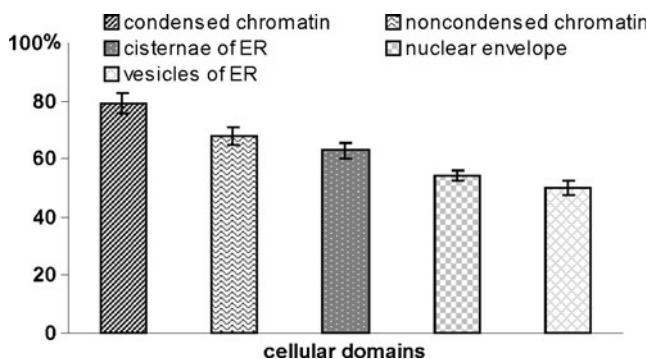| Compartment	P+CRT	P
	Grains [%]	Grains [%]
Endoplasmic reticulum		
Cisternae	5±0.9	3±0.45
Vesicles	14±0.98[a]	14±0.52[a]
Total	19±0.82	17±0.34
Whole nucleus	44±0.48	16±0.35
Nuclear envelope	4±0.24	3±0.1
Chromatin		
Condensed	27±1.53	7±0.96
Noncondensed	13±0.41	6±0.5
Total	40±0.79	13±0.52
Cytoplasm and other organelles	1±0.3	3±0.42

The number of gold grains in particular *C. vulgaris* spermatid compartments is represented as percentage of the whole pool of grains regarded as 100%; differences between percentage of protamine-type protein colocalization with calreticulin and protamine-type protein alone in the studied compartments are statistically significant taking $p \leq 0.05$ as the significance level; P+CRT - protamine-type proteins with CRT; P - protamine-type proteins without CRT

P+CRT protamine-type proteins with CRT, *P* protamine-type proteins without CRT

[a] These differences are not statistically significant

for example, with regard to protein abilities, showing that the animal sorting machinery was also functional for plant proteins. The researchers proved that basic CRT functions were conserved between the two kingdoms.

In plants, primary CRT localization was observed in ER lumen (Denecke et al. 1995; Napier et al. 1995; Opas et al. 1996; Lenartowska et al. 2002; 2009; Nardi et al. 2006). The present studies on *C. vulgaris* spermiogenesis resulted in the identification of the time and site of CRT presence. It was found to be the most abundant in ER at the onset of the exchange of nucleohistones into nucleoprotamines which is

extracts made of *C. vulgaris* antheridiostans at the V stage of spermiogenesis also showed CRT (55 kDa) and its isoform (62 kDa). Some scientists using Western blot technique confirmed that CRT isoforms fall in the range of 48–62 kDa in different organisms including plants (Gonzalez et al. 2002; Lenartowska 2009). Jia et al. (2009) noted that different isoforms of CRT might evolve to have some structural specificity favoring their targeting to different cellular parts for their respective functions. Postranslational modifications such as glycosylation, degradation of C-domain, and enzymatic modification of the glycan could mediate redistribution of CRT to other cellular compartments (Jethmalani et al. 1997; Navazio et al. 2002; Persson et al. 2003).

The use of animal antibodies against CRT in the studies on *C. vulgaris* was justified as Christensen et al. (2008) revealed that plant CRTs could substitute for animal CRTs,

Fig. 5 The percentage of protamine-type protein colocalization with CRT within particular *C. vulgaris* spermatid compartments at V stage of spermiogenesis. Gold grains from an individual compartment comprise 100%

indispensable for chromatin condensation and leads to the appearance of extremely condensed chromatin in mature spermatozoids.

Proper functioning of ER as a multifunctional system involved in protein transport is conditioned by various factors including the regulating mechanisms which control ER retention of the proteins, which are later transported and modified, and of chaperons, e.g., CRT, as well as of the enzymes conditioning protein maturation process. This involves coordinated synthesis of the cooperating components including those engaged in protein translocation. Unfortunately, little is known about the coordination of various phases of changes regarding ER (Stevens and Argon 1999; Chevet et al. 2001).

In ER lumen, there are numerous dissolved enzymatic proteins and molecular chaperons referred to as reticuloplasmins including immunoglobulin heavy-chain-binding protein BIP, protein disulphide isomerase PDI and CRT (Bedard et al. 2005; Hebert and Molinari 2007; Michalak et al. 2009).

In ER, chaperons transiently bind to non-folded or not fully folded proteins preventing their aggregation. Moreover, they slow down the folding process and increase the amount of properly folded proteins. CRT is involved in folding de novo synthesised proteins and glycoproteins (Michalak et al. 1999; Trombetta 2003). Present electron microscopic observations suggested that CRT could participate in translocation of de novo synthesized protamine-type proteins to a spermatid nucleus in *C. vulgaris* at the crucial V stage when the rearrangement of somatic nuclear proteins into generative ones started. It was revealed by a clearly noticeable colocalization of protamine-type proteins and CRT in a nucleus, nuclear envelope, and in extensive system of ER cisternae and vesicles which transport these proteins to a nucleus. Colloidal gold labelling coming from both antibodies, anti-CRT and anti-protamine-type proteins, during the V stage, significantly cumulated in a nucleus, especially at its periphery where condensed chromatin was present. Protamine-type protein labelling in this region was twofold greater than in the nuclei center filled with noncondensed chromatin (Popłońska et al. 2009). Moreover, in all studied cellular compartments, the percentage of protamine-type proteins/CRT colocalization was at least slightly or even significantly higher as compared to the protamine-type proteins alone. In addition, Lenartowska et al. (2001, 2009) clearly showed that rough ER was the site of CRT translation similarly as in the case of *Chara* protamine-type proteins (Popłońska et al. 2009).

In plants, e.g., in tobacco, it was observed that CRT stably complex with BIP was involved in protein folding (Crofts et al. 1998). CRT together with calnexin (CNX, a membrane chaperon similar to CRT) and Erp57 protein (PDI-type protein located in ER) constituted a CRT/CNX cycle which controlled quality and assembly of de novo synthesized proteins and glycoproteins (Trombetta 2003). Now it appears that complexes between ER-resident proteins including CRT could play a far more important role in protein folding (Gelebart et al. 2005). Confirming this, several chaperons, such as CNX1, CNX2, and SHEPHERD, were identified to highly coexpress with *Arabidopsis* CTRs (AtCTR1 and AtCRT2) (Obayashi et al. 2007), indicating that a tightly knit network of chaperon-like proteins may work together for protein folding in higher plants (Jia et al. 2009). Taking the above into consideration, as well as the present studies concerning alga spermiogenesis, we can suspect that CRT may be a chaperon involved in assembly and folding of de novo synthesized proteins in the studied species.

CRT also participates in reproductive process both in plants and animals. Enhancement of CRT expression was detected in zygotes and immature embryos of maize (Dresselhaus et al. 1996), in *Arabidopsis* flowers (Nelson et al. 1997), in maize sperm cells (Williams et al. 1997), and in tobacco anthers and pollen tubes (Nardi et al. 2006). CRT in *Petunia* can be involved in pollen–pistil interaction in vivo (Lenartowska et al. 2001). Moreover, immunoblot analysis of protein extracts from mature anthers, dry and germinated pollen, growing pollen tubes, and unpollinated/pollinated pistils revealed a strong expression of this chaperone (Lenartowska et al. 2009).

In animals, e.g., in mice and also in humans, two isoforms of CRT were revealed; one of them is strongly expressed in male reproductive organs—testes (Persson et al. 2002). This protein was also identified in human sperm neck (Naaby-Hansen et al. 2001), bull sperm neck (Ho and Suarez 2003), and in rat acrosome—both in spherical spermatids and in mature spermatozoids (Nakamura et al. 1993). It is worth noting that immunolabelling was significantly pronounced both in acrosome and in Golgi apparatus. It was observed that, during spermiogenesis in rat, CRT might be incorporated into acrosome via Golgi apparatus without glycosylation and might play an important role in spermatozoid function regulation, including their movement and in acrosome reaction (Nakamura et al. 1993). In *Chara,* despite the lack of acrosom, labelling of dictiosomes and Golgi vesicles was also observed. It is not surprising since, in plants, CRT was found in these cellular compartments earlier (Borisjuk et al. 1998; Navazio et al. 2002; Nardi et al. 2006). Moreover, in *Chara* colocalization of protamine-type proteins and CRT was observed in this organelle. Further studies will concentrate at elucidating the role of Golgi apparatus during *Chara* spermiogenesis.

Data from literature, as well as the present observations, support that CRT may take part in various biological processes, including spermiogenesis, both in animals and in plants.

Conflict of interest The author declares that she has no conflict of interest.

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