A homologue of the human regulator of mitotic spindle assembly protein (RMSA-1) is present in crane fly and is associated with meiotic chromosomes

Jing-Ping Yeo¹, Arthur Forer² and Ban-Hock Toh¹,*

¹Department of Pathology, Monash University Medical School, Commercial Road, Prahran, Victoria 3181, Australia
²Biology Department, York University, Downsview, Ontario M3J 1P3, Canada

*Author for correspondence

SUMMARY

In a previous study, we have shown that a newly identified chromosomal protein, RMSA-1 (Regulator of Mitotic Spindle Assembly-1), identified and cloned using a human autoimmune serum, is essential for mitotic spindle assembly; we proposed that RMSA-1 was a previously unknown physiological substrate for cdc2 kinase. In the present study, we show that this protein is present in crane fly and is associated with the chromosomes of spermatocytes.

A 31 kDa molecule in extracts from crane-fly nuclei, isolated from larvae, pupae and adults, reacts with affinity-purified anti-RMSA-1 autoantibody, shown by immunoblotting. The autoantibody reacts, as shown by immunofluorescence, with crane-fly spermatocyte chromosomes in prophase through anaphase of both meiosis-I and meiosis-II but does not react with preprophase or telophase nuclei or with spermatid nuclei. In all meiotic stages, the crane-fly sex chromosomes stain more intensely than the autosomes. We conclude that, since RMSA-1 is present in insect and mammalian cells, it is conserved across a variety of animal species. Further, since RMSA-1 binds to chromosomes in meiotic cells, it also may be essential for assembly of the meiotic spindle.

Key words: meiosis, spindle assembly, crane flies, mitosis

INTRODUCTION

Mitotic spindle assembly is an essential prerequisite for chromosomal segregation during mitosis. Much has been learnt about the mechanochemical properties of the spindle (reviewed by Wadsworth, 1993), but not much is known about the regulation of its assembly (reviewed by Norbury and Nurse, 1992). cdc2 kinase appears to play a key role in regulating spindle assembly, since sperm nuclei will promote spindle assembly in extracts of Xenopus zygotes only after addition of maturation promoting factor (which contains cdc2 kinase) (Lohka and Maller, 1985). Further, the interphase-metaphase transition of microtubule arrays is under cdc2 kinase control and requires phosphoproteins (Verde et al., 1990).

Spindle assembly requires centrosomes, kinetochores and chromatin. Centrosomes are required for microtubule nucleation (Karsenti and Maro, 1986; Kalt and Schliwa, 1993) while kinetochores are required to attach microtubules to chromosomes (Wadsworth, 1993). However, centrosomes per se are not universally necessary, since plant cells do not have them and centrosomes can be separated from meiotic spindle poles in crane-fly spermatocytes (Dietz, 1966; Steffen et al., 1986). The role of chromatin in promoting spindle assembly is supported by the following observations. Firstly, ultrastructural studies have shown that mitotic spindle assembly induced in Xenopus eggs by injection of sperm nuclei is not dependent on kinetochore-microtubule interactions (Sawin and Mitchison, 1991). Secondly, anti-centromere autoantibody, microinjected into cells, interferes with kinetochore assembly and chromosomal movement, but fails to inhibit mitotic spindle formation (Bernat et al., 1990, 1991; Simerly et al., 1990). Thirdly, spindle-like microtubule arrays form around condensing chromatin when karyoplasts, free of centrosomes, are injected into the cytoplasm of metaphase Xenopus eggs (Karsenti et al., 1984). Finally, removal of chromosomes from grasshopper spermatocytes reduces the number of kinetochore and non-kinetochore microtubules (Nicklas and Gordon, 1985).

We have suggested that RMSA-1 (regulator of mitotic spindle assembly-1), a new 47 kDa chromosomal protein identified and cloned using an autoimmune serum, may be such a chromatin factor that promotes mitotic spindle assembly because anti-sense mRNA or autoantibody prevented spindle formation (Yeo et al., 1994). Further, we have proposed that RMSA-1 may be a physiological substrate for cdc2 kinase. In the present article, we show that RMSA-1 is activated by phosphorylation by cdc2 kinase at the G2/M transition, and that activated RMSA-1, alone or as a complex, promotes mitotic spindle assembly.

In the present article, we show that RMSA-1 is a 31 kDa protein in crane fly, that it is present along the entire length of each crane-fly meiotic chromosome, and that crane-fly sex chromosomes stain (shown by immunofluorescence for RMSA-1) more intensely than autosomes. These observations
suggest that RMSA-1 is conserved in evolution and that RMSA-1 also may be required for meiotic spindle assembly.

MATERIALS AND METHODS

Sera

The autoimmune serum, from a patient with discoid lupus erythematosus, was identified during routine screening for anti-nuclear autoantibodies at Gribble’s Pathology, Melbourne, Australia. The autoimmune serum and control normal human serum were heat inactivated at 56°C for 30 minutes and stored at −70°C until required. Affinity-purified autoantibodies were prepared as described (Yeo et al., 1994). Briefly, autoantibody was eluted from fusion protein expressed by recombinant phages and IgG-purified by chromatography on Protein G-Sepharose. Normal human IgG was also similarly Protein G-selected.

Crane-fly spermatoocytes

Spermatocytes were prepared from crane flies (Nephrotoma ferruginea Fabr.) reared in the laboratory. Rearing and cell preparation procedures are described in detail elsewhere (Forer, 1982). Briefly, we covered animals with Halocarbon oil (Halocarbon Inc., Hackensack, New Jersey), removed the testes (under oil) and placed them in a drop of insect Ringer’s solution. (Insect Ringer’s solution is: 0.13 M NaCl, 0.05 M MgCl₂, pH 7.4). For isolation of nuclei, the homogenate was suspended in PBS containing protease inhibitors (1 mM PMSF, 1 mM pepstatin, 2 µM EDTA, 0.5 mM DTT, 15% (v/v) glycerol, 0.5 mM PMSF, 0.5 µM pepstatin, 2 µg/ml leupeptin, pH 8.0) containing 1 M NaCl or 0.5 % NP40. With nuclei extracted with 1 M NaCl, the residual nuclear debris was pelleted in TM buffer containing 2.2 M sucrose and centrifuged at 70 °C until required. finalists were eluted with the autoimmune serum at 56 °C for 30 minutes and stored at 4 °C until required.

Subcellular fractionation

Crane-fly larvae, pupae and adults were washed 3× at room temperature in PBS (0.145 M NaCl, 0.01 M phosphate, pH 7.2), and resuspended in PBS containing protease inhibitors (1 mM PMSF, 1 mM leupeptin and 65 µg/ml aprotinin). Total cell extracts were prepared by homogenising crane-fly tissues (larvae, pupae and adults) in 2 volumes of TM buffer (0.25 M sucrose, 0.05 M Tris-HCl, pH 6.8) containing protease inhibitors (1 mM PMSF, 1 mM tris-HCl, 0.05 M MgCl₂, pH 7.4). For isolation of nuclei, the homogenate was filtered through cheese cloth and centrifuged at 770 g for 10 minutes (Sorvall SS34 rotor) to yield a crude nuclear pellet. The supernatant was kept as the cytosolic extract. The crude nuclear pellet was resuspended in TM buffer containing 2.2 M sucrose and centrifuged at 40,000 g for 1 hour (Beckman 21 rotor). The pellet, washed twice in TM buffer and resuspended in SDS-PAGE sample buffer, was kept as the nuclear fraction. For preparation of nuclear extracts, isolated nuclei were resuspended in extraction buffer (20 mM HEPES, 0.5 mM EDTA, 0.5 mM DTT, 15% (v/v) glycerol, 0.5 mM PMSF, 0.5 µg/ml leupeptin, pH 8.0) containing 1 M NaCl or 0.5 % NP40. With nuclei extracted with 1 M NaCl, the residual nuclear debris was pelleted at 13,000 g for 1 minute and the supernatant kept as the nuclear extract. Protein concentration of each sample was estimated using the BCA protein assay kit (Pierce Chemical Co. USA).

Immunoblotting

Total extracts, nuclear and cytosolic fractions, and nuclear extracts, of crane-fly larvae, pupae and adults were immunoblotted with affinity-purified anti-RMSA-1 autoantibody. Proteins (100 µg/lane) were separated by discontinuous SDS-PAGE under reducing (5% 2-β-mercaptoethanol) or non-reducing conditions, transferred to nitrocellulose using a semi-dry blot system (Hoeffer) for 1 hour at constant current (0.8 mA/cm² of gel) and reacted by immunoblotting with the autoimmune serum or with the affinity-purified autoantibody as described (Yeo et al., 1994).

Immunofluorescence

We processed coverslips for immunofluorescence in one of two ways. (1) Cells were lysed with an NP-40-containing microtubule-stabilizing medium, fixed with 0.5% glutaraldehyde for 10 minutes, treated with borohydride, and reacted with the autoimmune serum (see Czaban and Forer, 1992, for details). These cells did not bind the autoimmune serum but cells treated this way bound antibody to tubulin. (2) The cells were sequentially treated with 2% parafomaldehyde in insect Ringer’s solution for 5 minutes (or, in a few instances, without fixative in PBS); 1% NP40 in 2% parafomaldehyde in insect Ringer’s solution for 5 minutes; and with 1% NP40 in PBS for 10 minutes. Then the cells were rinsed with PBS twice and either stained immediately or stored at 4°C in a 1:1 mixture of PBS: glycerol until they were stained. We report here on cells treated by procedure (2).

For immediate staining, the coverslips were incubated with autoimmune serum (diluted 1:200 in PBS) or with affinity-purified autoantibody (1 µg), for 30 minutes at room temperature under humidified conditions, and processed for immunofluorescence microscopy as described (Yeo et al., 1994). The cells were washed three times in PBS, counterstained with Hoechst 33342 DNA dye (5 µM in PBS) and mounted in glycerol/PBS. For preparations stored in glycerol/PBS, glycerol was first replaced with PBS. Then the cells were incubated for 30-45 minutes with autoimmune serum diluted 1:100 in PBS, rinsed 3 times with PBS, incubated for 30-45 minutes with FITC-labelled anti-human IgG, rinsed 2 times with PBS, and mounted in Mowiol (Rodriguez and Deinhardt, 1960; Osborn and Weber, 1982) to which was added 0.2 g/l PPD (p-phenylendiamine), to retard fading (Platt and Michael, 1983; Storz and Jelke, 1984). For control experiments, all the steps were the same as above except that the initial incubation was with normal human serum or with normal human IgG (1 µg).

We studied the fluorescent images using a Leitz microscope and a x50, NA 1.3 objective lens (Melbourne) or a Nikon Optiphot microscope (using a x60, NA 1.4 lens) that made up part of a Bio-Rad MRC-600 confocal microscope (Toronto). Images were obtained either by photographing through the Leitz microscope (using Kodak Ektachrome film) or with a video printer (Mitsubishi P40U) from video images displayed by the confocal microscope. The illustrations from confocal images are of superposed images: the individual images in the z-series from each cell were superposed via the computer to give a composite picture.

We quantified maximum grey scale values of each chromosome from the superposed z-series images: we moved a cursor in the entire region of the chromosome and recorded the maximum grey scale value. For this analysis, we used only images in which the superposed chromosomes did not lie over each other.

RESULTS

Immunoblots: RMSA-1 nuclear protein is present in crane-fly cells

We have previously shown that human anti-RMSA-1 autoantibody reacts with a 47 kDa protein in immunoblots of mammalian nuclear extracts (Yeo et al., 1994). In the present study, affinity-purified anti-RMSA-1 autoantibody reacted in immunoblots with a 31 kDa protein in crude crane-fly extracts under reducing conditions (Fig. 1A) or non-reducing conditions (data not shown). The 31 kDa protein was detected throughout the insect’s developmental stages from larvae to pupae to adults. No immunoreactive protein was detected with normal human IgG.

To determine if the protein is in the nucleus as is the case with mammalian and avian species (Yeo et al., 1994), nuclei were isolated from the insect by two methods. The first method
was to prepare nuclei and nuclear supernatant in which the nuclear envelope remained intact. Preparations were counterstained with Hoechst DNA dye to determine whether they contained nuclei. The second approach was to prepare nuclei and nuclear supernatant in which the nuclei had been permeabilised by treatment with the detergent NP40. Immunoblotting of both intact (Fig. 1B, lanes 4-6) and permeabilised (Fig. 1C, lane 3) nuclear preparations confirmed that the RMSA-1 protein is in the nuclei, not in the cytosol. Therefore, the 31 kDa protein is a bona fide resident of the nucleus in crane-fly cells. Further, studies with mammalian nuclei have shown that mammalian RMSA-1 can be extracted with salt concentrations up to 1 M NaCl. Similarly, insect RMSA-1 can also be extracted from nuclei with 1 M NaCl (Fig. 1C, lane 2).

**Immunofluorescence: RMSA-1 is associated with crane-fly spermatocyte meiotic chromosomes**

We have previously shown that in mammalian cells (human Hep-2 and HeLa cells, monkey COS cells, mouse 3T3 and Ltk cells, and hamster CHO cells) anti-RMSA-1 autoantibody reacts, by immunofluorescence, only with condensed mitotic chromosomes, at all stages from prophase through telophase, but does not react with interphase cells. Similar results were obtained with crane-fly meiotic spermatocytes, as follows.

In meiosis-I, crane-fly spermatocytes contain three autosomal bivalents and two univalent sex chromosomes. At anaphase, the autosomes separate into half-bivalents, which move poleward, but until the autosomes reach the poles the sex chromosomes remain at the equator, each with a spindle fibre to each pole, as illustrated after anti-tubulin staining in Fig. 2. The sex chromosomes move to opposite poles only after the autosomes reach the poles. The positions of the chromosomes change at different stages of meiosis-I, so one can determine the stage of any given cell from the positions of each of the chromosomes (e.g. see descriptions by Dietz, 1969 or Forer, 1980), and one can distinguish meiosis-I from meiosis-II by both the numbers and kinds of chromosomes in the cell.

Condensed chromosomes in crane-fly spermatocytes reacted with anti-RMSA-1 autoantibody at all stages from prophase to telophase, in both meiosis-I and meiosis-II; no other organelle was fluorescent, and no stages before prophase-I or after telophase-II reacted with the autoantibody. These observations are illustrated in Fig. 3, in which two different fields of view are compared, as seen with autoantibody staining and Hoechst staining. No fluorescence was seen with parallel slides incubated with control serum. Therefore, we conclude that, under the immunostaining conditions employed, condensed chromosomes react with anti-RMSA-1 autoantibody but nuclei do not.
Fig. 3. A comparison of two different fields of view seen using fluorescence from the Hoechst stain (A and B) or from the staining of RMSA-1 (C and D). (A and C) One field of view; (B and D) a different field of view; both of these were photographed using the Leitz microscope. In each field of view only one cell is stained with the anti-RMSA-1 antibody. In each case, a cell is shown in late anaphase with the two sex chromosomes near the equator (but moving poleward) and the autosomes clumped near the poles. Pre-prophase nuclei and spermatid nuclei stain with the Hoechst stain but not with anti-RMSA-1 antibody. ×2900.
The RMSA-1 antigen is present along the entire length of each chromosome. This can be seen in individual confocal images, especially when the individual confocal images are superposed and the chromosomes are separate spatially (e.g. in prophase in Fig. 4C and in prometaphase in Fig. 4D). The confocal images were recorded under conditions in which the staining is with anti-RMSA-1 antibody. The two sex chromosomes are brightly fluorescent and are indicated by arrows in C. Presumed sex chromosomes are shown in A and B and are indicated by arrows in A, the only stained material in this very early prophase nucleus, ~×1700. (D-G) Meiosis-I prometaphase (D), metaphase (E), early anaphase (F), and mid-anaphase (G) crane-fly spermatocytes as seen using the confocal microscope after z-series images were superposed. Staining was with anti-RMSA-1 antibody. The two sex chromosomes are indicated by arrows in D-G; the only other components stained are the autosomes. D, ~×1500; E and F, ~×1800; G, ~×2300. (H-J) Meiosis-I late anaphase (H,I) and telophase (J) crane-fly spermatocytes as seen using the confocal microscope after z-series images were superposed. The staining is with antibody against RMSA-1. The two sex chromosomes are indicated by arrows in all three illustrations. The only other components stained are the autosomes. ~×1700. (K-M) Meiosis-I prophase (K,L) and prometaphase (M) crane-fly spermatocytes as seen using the confocal microscope after z-series images were superposed. Staining is with antibody against RMSA-1; the chromosomes seem non-homogeneously stained (some brighter regions are indicated by arrows). L and M are illustrated at lower magnification in C and D, respectively. K, ~×4000; L, ~×2400; M, ~×2200.
maximum fluorescence did not saturate the camera (i.e. all grey scale values were between 0 and 256), and our clear visual impression is that the sex chromosomes fluoresced more brightly than the autosomes, in all stages of meiosis-I (e.g. see Fig. 4C-J). The sex chromosomes fluoresced earlier in prophase than did the autosomes (e.g. see Fig. 4A and B), and retained their fluorescence in later division stages than did the autosomes (e.g. see Fig. 4I). Further, one of the two sex chromosomes appeared to fluoresce less intensely than the other, especially in earlier and later stages of division (Fig. 4A,I,J), though both fluoresced more intensely than autosomes.

We quantified the fluorescence intensities of the confocal images, to test whether our visual impressions were correct. As listed in Table 1, our visual impressions indeed were accurate: the sex chromosomes were more brightly fluorescent than the autosomes, and the two sex chromosomes were of different fluorescence intensity.

The fluorescence intensities did not often seem to be uniform along the lengths of the autosomes or sex chromosomes (e.g. see Fig. 4C,D,F); several examples are illustrated at somewhat higher magnification in Fig. 4K-M. This is a consistent impression, verified by intensity scans along the lengths of the chromosomes. However, it is difficult to determine whether this is due to differential chromosome packing, or to differential effects of the fixation/staining regime. Nonetheless, we very often noticed non-uniform staining, and it may be that this indicates that the antigen is non-uniformly distributed along the bodies of chromosomes.

**DISCUSSION**

We have shown that crane-fly spermatocytes contain a 31 kDa protein specifically recognised by anti-RMSA-1 autoantibody and that this protein is associated with condensed meiotic chromosomes. The same serum binds to a 47 kDa mammalian protein required for mitotic spindle assembly (Yeo et al., 1994). Since RMSA-1 is associated with chromosomes in both divisions of spermatocyte meiosis in crane flies, but not with interphase cells, we suggest that crane-fly RMSA-1 may also be necessary for meiotic spindle assembly.

The crane-fly spermatocyte sex chromosomes stained more intensely than autosomes. In mammalian cells, the levels of RMSA-1, detected by immunoblotting, remained relatively constant throughout the cell cycle, indicating that absence of immunofluorescence in interphase cells is not due to protein degradation, unlike the case of cyclin B (Norbury and Nurse, 1992) and CENT-E (Yen et al., 1992). These observations suggest that in interphase cells the nuclear epitope may be masked, perhaps as a result of a change in phosphorylation that we have previously reported with mammalian RMSA-1 (Yeo et al., 1994). The results from staining crane-fly spermatocytes, then, might mean that the RMSA-1 protein associated with sex chromosomes is more highly phosphorylated than the protein associated with autosomes. Sex chromosome arms in this species of crane fly are not much longer than the kinetochore (Fuge, 1974); since the sex chromosomes have spindle fibres to both poles, the kinetochores are present on both sides of the chromosome, as illustrated in Fig. 2. Another explanation may be that this kinetochore arrangement requires more phosphorylated RMSA-1 than the usual arrangement.

RMSA-1 may be a physiological substrate for cdc2 kinase, an enzyme that plays a pivotal role in the regulation of the eukaryotic cell cycle (reviewed by Norbury and Nurse, 1992; Murray, 1992). This kinase, active at the G2/M transition, is thought to phosphorylate key molecules that mediate events associated with mitosis. Therefore, it is important to identify the physiological substrates phosphorylated by this enzyme and to identify the mitotic events regulated by these phosphorylated substrates. However, only a few candidate substrates for cdc2 kinase that are linked to mitotic events have been established. These events include phosphorylation of the nuclear lamins that regulate nuclear lamina breakdown (Peter et al., 1990; Heald and Mckeon, 1990; Ward and Kirschner, 1990) and phosphorylation of cytoskeletal proteins vimentin and caldesmon, which are associated with reorganisation of intermediate filaments (Chou et al., 1990) and microfilaments, respectively (Yamashiro et al., 1991). The cdc2 kinase substrates that regulate other mitotic events including chromosome condensation and mitotic spindle assembly remain largely unknown (reviewed by Norbury and Nurse, 1992; Murray, 1992). Based on comparison of putative cdc2 kinase phosphoacceptor sites in histone H1 and p60src, a motif of S/T-P was proposed as a consensus sequence for phosphorylation by cdc2 kinase (Shenoy et al., 1989). Since RMSA-1 is phosphorylated only in mitotic cells and contains three S/T-P consensus motifs, it may serve as a physiological substrate for cdc2 kinase (Yeo et al., 1994). If so, then one would expect the same substrate to be conserved across phyla, as is the case for cdc2 kinase. The results we present here support this suggestion, by showing that a similar RMSA-1 protein is associated with crane-fly spermatocytes.

Autoantibodies to ‘chromosomal passengers’, a group of ‘migratory’ proteins associated with chromosomes during prometaphase and metaphase, and which are relocated to the spindle midzone at anaphase, have been described (reviewed by Earnshaw and Bernat, 1991). However, RMSA-1 is distinct from these chromosomal passengers, since it is associated only with mitotic and meiotic chromosomes. The latter observation also distinguishes RMSA-1 from centromere autoantigens associated with interphase and mitotic chromosomes (Earnshaw et al., 1987). However, the distribution of RMSA-1 along the entire length of mitotic and meiotic chromosomes may also include the centromere.

The ability of the human autoantibody to react with RMSA-1 antigen in crane fly, species separated by billions of years of evolution, is typical of these reagents, which have proven to be powerful tools for studies of the molecular cell biology of...
the nucleus; and, indeed, the autoantibodies often bind to active sites of nucleosomes (reviewed by Tan, 1989). For instance, centromere antigens CENT-B and CENT-C, and NuMa, a nuclear mitotic apparatus protein, have been cloned using autoantibodies (Cooke et al., 1990; Saitoh et al., 1992; Yang et al., 1992). In addition to providing structural information, autoantibodies are also useful for elucidating the function of nuclear components. For instance, autoantibodies to Sm/nRNP autoantigens were critical for defining these molecules as spliceosome components in pre-mRNA splicing (Maniatis and Reed, 1987).

In summary, we propose that RMSA-1, associated with mitotic or meiotic chromosomes, is activated by phosphorylation at the G2/M transition by cdc2 kinase and that activated RMSA-1 regulates spindle assembly in both mitosis and meiosis by stabilising spindle microtubules (Kirschner and Mitchison, 1986; Karsenti, 1991; Mitchison, 1992).

This work was supported by the Mary Whight Lupus Fellowship, the Anti-Cancer Council of Victoria and the Australian Research Council, and by the Natural Sciences and Engineering Research Council of Canada. We thank Mrs E. Smith for donation of blood samples and Dr Peter Fergin and staff at Gribble’s Pathology for assistance in obtaining these blood samples.

REFERENCES


Kirschner and Mitchison (1986).


Verde et al. (1990).


(Rceived 8 February 1994 - Accepted 22 March 1994)