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Microinjection of antibodies to centromere protein CENP-A arrests cells in interphase but does not prevent mitosis

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Abstract. Centromere protein CENP-A is a histone H3-like protein associated specifically with the centromere and represents one of the human autoantigens identified by sera taken from patients with the CREST variant of progressive systemic sclerosis. Injection of whole human autoimmune serum to the centromere into interphase cells disrupts some mitotic events. It has been assumed that this effect is due to CENP-E and CENP-C autoantigens, because of the effects of injecting monospecific sera to those proteins into culture cells. Here we have used an antibody raised against an N-terminal peptide of the human autoantigen CENP-A to determine its function in mitosis and during cell cycle progression. Affinity-purified anti-CENP-A antibodies injected into the nucleus during the early replication stages of the cell cycle caused cells to arrest in interphase before mitosis. These cells showed highly condensed small nuclei, a granular cytoplasm and loss of their division capability. On the other hand, microinjection of nocodazole-blocked HeLa cells in mitosis resulted in the typical punctate staining pattern of CENP-A for centromeres during different stages of mitosis and apparently normal cell division. This was corroborated by time-lapse imaging microscopy analysis of mid-interphase-injected cells, revealing that they undergo mitosis and divide properly. However, a significant delay throughout the progression of mitotic stages was observed. These results suggest that CENP-A is involved predominantly in an essential interphase event at the centromere before mitosis. This may include chromatin assembly at the kinetochore coordinate with late replication of satellite DNA to form an active centromere.

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Introduction

The kinetochore complex is a defined structure on the mitotic chromosome that adheres to the primary constriction at the centromere. The kinetochore serves as the site for attachment of spindle microtubules, which facilitate the alignment and separation of chromosomes during mitosis. Identification and characterization of molecular components of the kinetochore has provided the basis for a biochemical understanding of various events that occur at the complex during mitosis (Brinkley et al. 1992; Earnshaw and Tomkiel 1992; Choo 1997). In mammals, numerous components have been identified, either by recognition of human autoantibodies (Moroi et al. 1980; Guldner et al. 1984; Earnshaw and Rothfield 1985; Valdivia and Brinkley 1985; Kingwell and Rattner 1987; He et al. 1998), or by raising antibodies to purified antigens (Compton et al. 1991; Yen et al. 1991; Williams et al. 1992; Wordeman and Mitchison 1995). In some cases, human autoantigens, known as CENPs (centromere proteins) (Earnshaw and Rothfield 1985), have been characterized to provide evidence of their function at the centromere. Thus, CENP-B is an α-satellite DNA-binding protein (Masumoto et al. 1989; Sugimoto et al. 1992) located in the central heterochromatic region of the centromere, underlying the kinetochore plates (Cooke et al. 1990). CENP-B may play a role in the higher order folding of alphoid chromatin through self-assembly (Yoda et al. 1992). CENP-C is a component of the trilaminar kinetochore. Its location is restricted to the inner kinetochore plate at interphase, between the centromeric heterochromatin and the outer kinetochore plate, to which microtubules are attached (Saitoh et al. 1992). In vitro DNA-binding studies suggest that CENP-C binds to DNA (Yang et al. 1996). CENP-E is a microtubule-dependent motor integral component of kinetochore corona fibers that links centromeres to spindle microtubules (Yen et al. 1992; Schaar et al. 1997). CENP-F is a protein of the nuclear matrix that assembles onto kinetochores at the outer plate at late G2-phase and is rapidly degraded after mitosis (Rattner et al 1993; Liao et al. 1995). CENP-G is a new

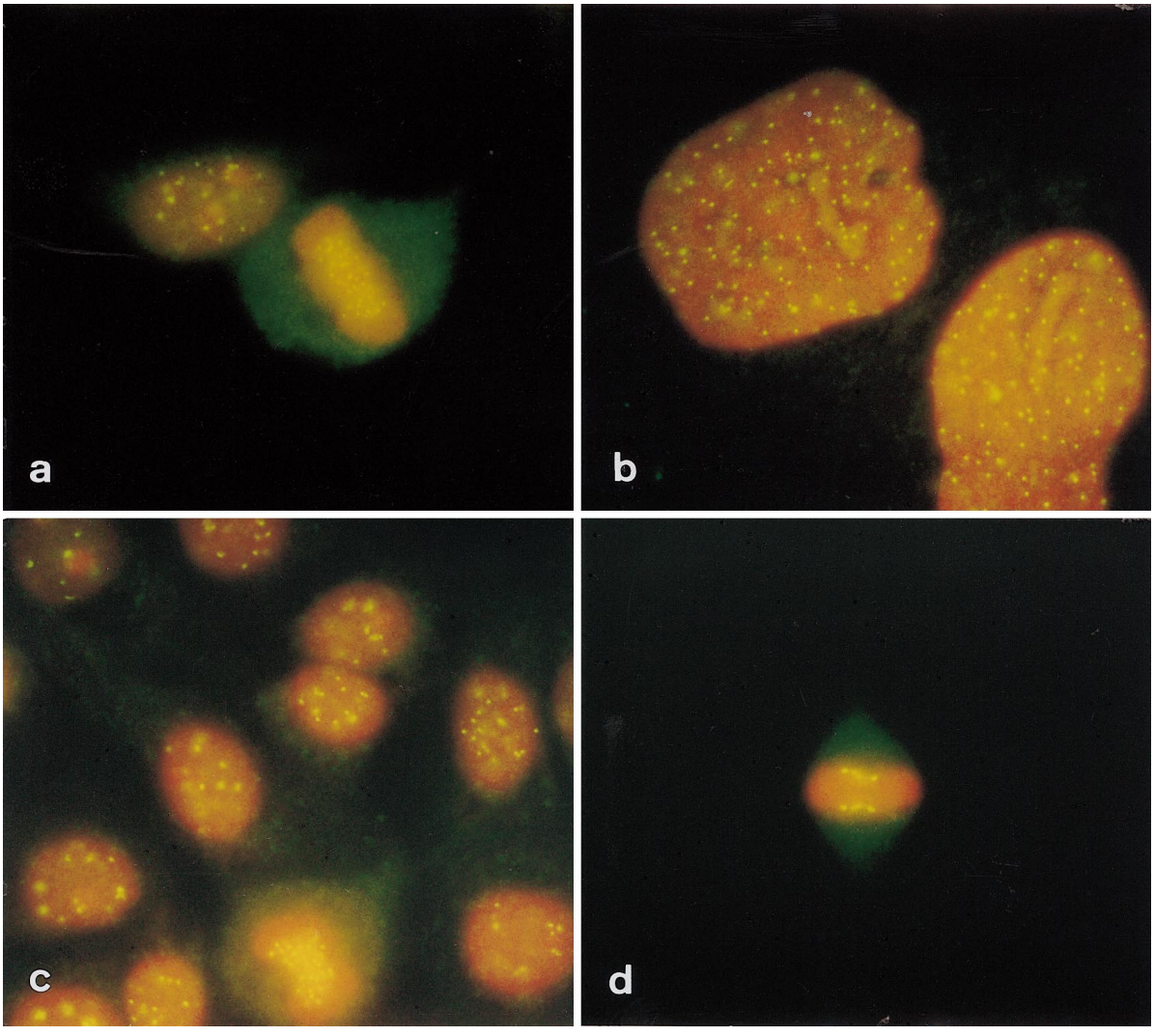


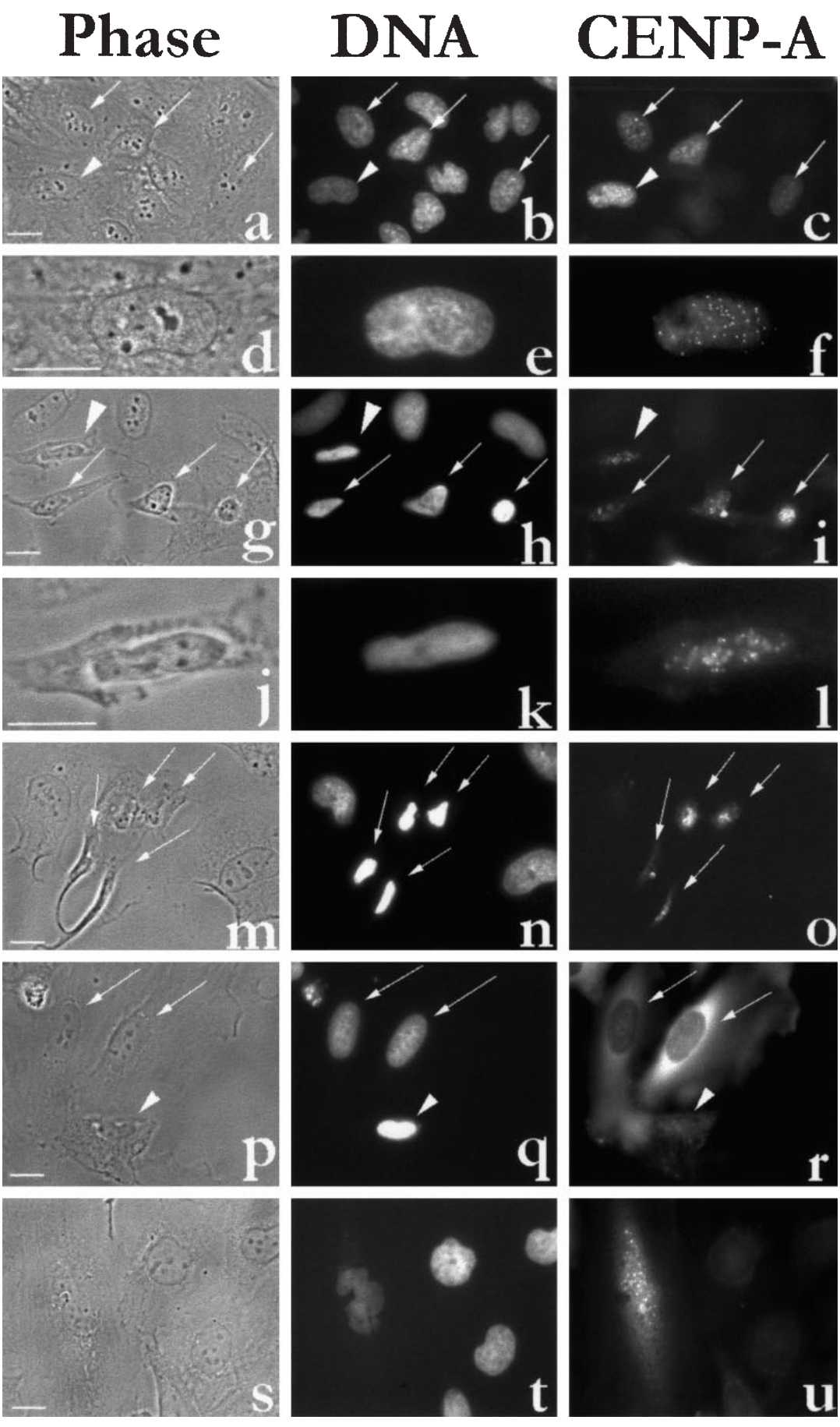
Fig. 1a–d. Immunofluorescence staining of mammalian culture cells with specific anti-CENP-A serum. Staining is restricted to a punctate pattern at the centromeres in all stages of the cell cycle. The N-terminal CENP-A epitope (amino acid residues 3-17) is conserved in HeLa (**a**), CV-1 (**b**), MDBK (**c**) and Indian muntjac (**d**) centromeres. Original magnification 100×

protein detected at the centromeric region that is restricted to the kinetochore inner plate in mitosis. This protein was shown to be associated with an α-1 satellite DNA subfamily at the centromere (He et al. 1998). Finally, CENP-A is a histone H3 variant concentrated in the region of the inner kinetochore plate of active centromeres (Palmer et al. 1987; Sullivan et al. 1994; Warburton et al. 1997).

Microinjection of whole human centromere antibodies has been demonstrated to disrupt events required for chromosome movement at mitosis (Bernat et al. 1990; Wordeman et al. 1996). This was approached accurately in some cases by injecting specific antibodies to CENP-C (Tomkiel et al. 1994) and CENP-E (Yen et al. 1991; Schaar et al. 1997), and it has been possible to assign specific functions to those autoantigens in mitosis.

The purpose of the present study was to determine the effect of microinjection in HeLa cells of monospecific antibodies generated against CENP-A (Valdivia et al. 1998), to investigate the role of this centromere-associated protein in kinetochore assembly and function.

Fig. 2a–u. Analysis of HeLa cells microinjected with affinity-purified anti-CENP-A antibodies. Cells were synchronized in G1/S-phase by double blocking with thymidine, released for several hours, microinjected, and analyzed for CENP-A by using fluorescein-labeled secondary antibody. Cells are shown in phase contrast, 4',6-diamidino-2-phenylindole (DAPI) staining for DNA and fluorescence for CENP-A. Releasing from G1/S block was for 3 h (**a–o**) or 8 h (**p–u**) before microinjection. Analysis for fluorescence was at 30 min (**a–f**), 1 h 30 min (**g–l**), 22 h (**m**, **n**, **o**) and 1 h 30 min (P–4) after microinjection. Anti-CENP-A injected early (3 h) after release, produced arrest of cells in interphase with the accumulation of very condensed small nuclei (*arrows* in **h** and **n**) (compare with noninjected cells in the same plate), with a granular appearance of the cytoplasm (details of cells indicated by *arrowhead* in **a–c** and **g–i** are in **d–f** and **j–l**, respectively. This effect was first observed 1 h 30 min after the injection of anti-CENP-A into the cell (compare injected nuclei in **c** with those in **i** and **o**). However, cells injected later (8 h) after release, exhibited normal nuclei (**p**, **s**) as compared with noninjected cells, and CENP-A centromeric punctate staining (**r**, **u**). Cells arrested in interphase by anti-CENP-A (**i**, **o**) presumably failed to reach mitosis because of interference in some event in S/G2 that is not produced if microinjection of the anti-CENP-A antibody is performed late in S (**r**, **u**). *Bars* represent 10 μm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Materials and methods

Antibodies. Production and characterization of a CENP-A-specific antiserum to a synthetic peptide covering amino acid residues 3-17 of human CENP-A was as described in a previous report (Valdivia et al. 1998). For the microinjection experiments, this serum was affinity-purified in a CNBr-Sepharose column containing the covalently bound synthetic CENP-A peptide (produced by Eurogentec, Belgium). Purified immunoglubulins were dialyzed, concentrated to approximately 5 mg/ml and stored at –70°C until use. Several antibodies were used as controls, including preimmune serum from the same rabbit producing the anti-CENP-A antibodies, and a human CREST autoimmune serum, which detects CENP-A on immunoblots and contains a high titer of CENP autoantigens.

Cell culture and microinjection. HeLa cells were grown at low density in DMEM (Gibco Laboratories) with 5% fetal calf serum. In all cases, individual cells were microinjected using an Eppendorf system consisting of a 5171 micromanipulator and a 5242 microinjector. For the microinjection, cells were accumulated either at the G1/S boundary by a double thymidine block (2 mM) (Stein et al. 1994), or in early mitosis by nocodazole (0.04 μg/ml) for 4 h (Zieve et al. 1980). In an initial set of experiments, the cells blocked at G1/S were injected into the nucleus with anti-CENP-A at 1–12 h after release from the drug, then fixed at different times and processed for immunofluorescence by incubating with fluorescein-labeled secondary antibody. In some cases, the injected antibody was also found in the cytoplasm, probably indicating either initial cytoplasmic injection, degradation of the injected antibody or residual immunoglobulin left from mitotic cells in the cytoplasm after cell division. In a second set of experiments, HeLa cells synchronized at early mitosis with nocodazole were injected with anti-CENP-A, released immediately after injection and fixed and processed for immunofluorescence at different times (between 30 min and 12 h) after microinjection. In a final set of experiments we monitored living cells injected with anti-CENP-A, by time-lapse imaging microscopy. In this case, cells synchronized at the G1/S boundary were released from thymidine block and injected at different times with affinity-purified anti-CENP-A antibodies together with a small amount of Texas Red-conjugated Dextran (Molecular Probes, 0.5% final concentration in PBS) used as an indicator of injected cells (Pepperkok et al. 1988). The injection of either Texas Red solution, PBS-BSA, or rabbit preimmune serum did not produce any significant effect on cell viability and cells progressed normally through the complete cell cycle. Cells microinjected with human CREST centromere antibodies were also used as a control in time-lapse experiments (see later in Discussion). Observation was on a Zeiss Axiovert 10 inverted microscope equipped with a Photometrics CH250 digital cooled slow-scan CCD camera connected to a SUN workstation. Image acquisition, processing and animation of time-lapse series were done using the software package Khoros (Herr et al. 1993). 120–200 phase contrast images were acquired with a 20× objective every 10 min, corresponding to a total observation time of about 20–28 h after microinjection. Images were printed using Adobe Photoshop software.

Immunofluorescence. Mammalian cells were grown in DMEM in the presence of 7% fetal calf serum on coverslips until 75% confluence, before being used for anti-CENP-A staining analysis. Cells were fixed and permeabilized in methanol at –20°C for 10 min and incubated (only for results shown in Fig. 1) with primary anti-CENP-A antibody (dilutions 1:400 to 1:1,000) in PBS for 30 min at 37°C. Then the cells were washed three times with PBS and incubated for 30 min at 37°C with fluorescein-labeled secondary antibody (dilution 1:80) in PBS. Cells were washed again three times in PBS, counterstained with 4',6-diamidino-2-phenylindole (Sigma, 0,5 μg/ml) for DNA and mounted with PBS:glycerol (1:9). Images were taken using a Zeiss Axiophot fluorescence microscope photographed on T-max 100 film. Immunofluorescent images of microinjected cells were acquired as above.

Results

Monospecific anti-CENP-A serum recognizes a highly conserved epitope in mammalian centromeres

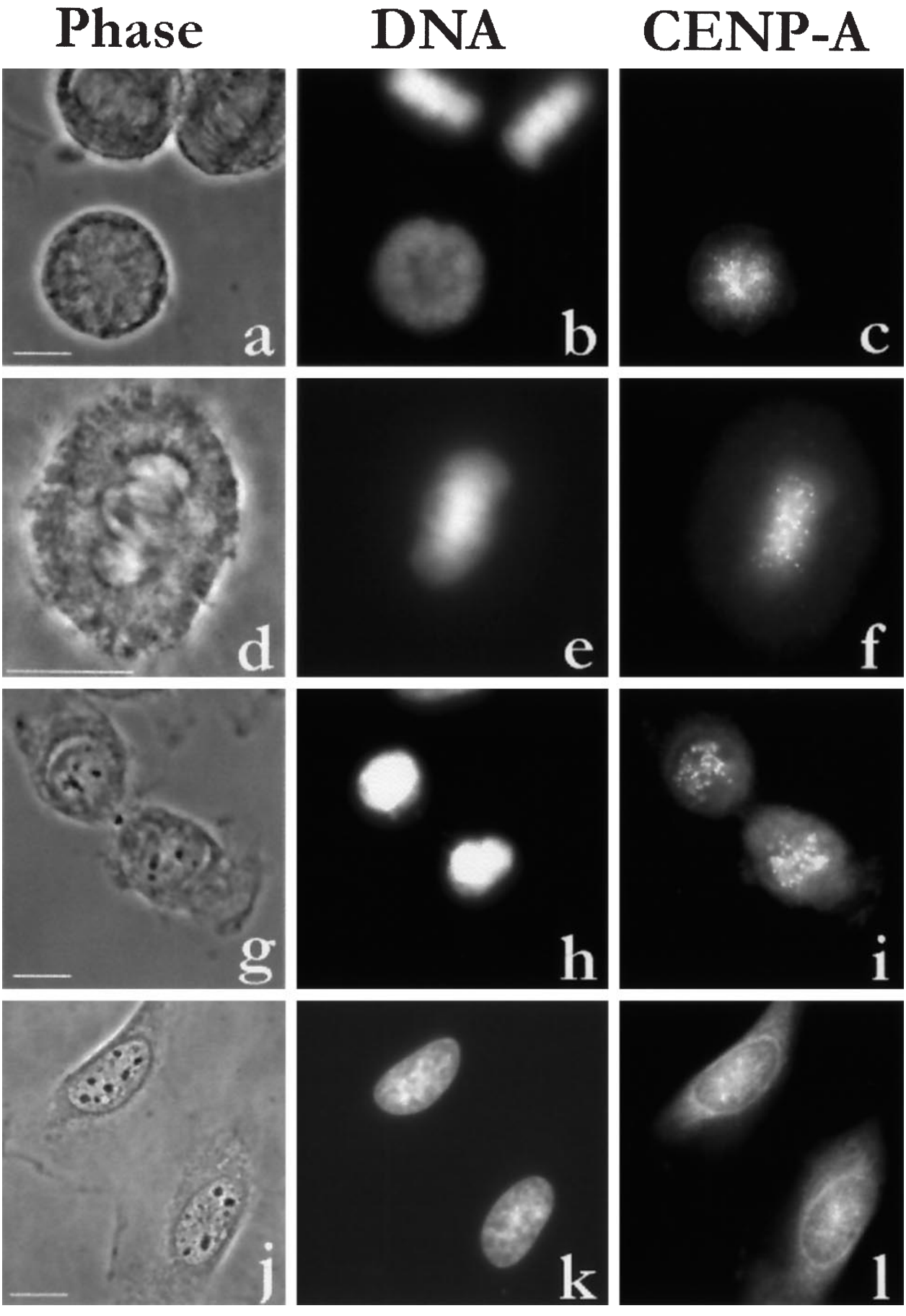
In a previous study we have described the immunological characterization of a new probe for the human centromeric autoantigen CENP-A (Valdivia et al. 1998). In that report, the serum was shown to recognize the M_r 18,000 centromeric autoantigen in protein extracts from nuclei of human placenta. Here, we have extended our findings and have shown the expression and epitope conservation of the N-terminal region of CENP-A in several mammalian cell lines, including human (HeLa), monkey (CV-1), bovine (MDBK) and deer (Indian muntjac). The specificity of the anti-CENP-A to the kinetochore is clearly seen by immunofluorescence throughout all stages of the cell cycle in the cell lines analyzed (Fig. 1). Visualization by immunofluorescence of the final N-terminal epitope of CENP-A (amino acids residues 3-17) in several mammalian cell lines may provide information on the conserved secondary organization of that region of the centromeric antigen at the kinetochore. Electron microscopy studies with this serum could definitively serve to locate CENP-A unequivocally at the inner plate of kinetochore, as suggested in a previous report based on immunofluorescence analysis (Warburton et al. 1997).

Microinjection of affinity-purified anti-CENP-A antibodies in G1/S-phase arrests HeLa cells in interphase

Microinjection of anti-CENP antibodies is a reliable method for interfering with CENP function at the centromere/kinetochore region (Bernat et al. 1990; Tomkiel et al. 1994; Wordeman et al. 1996; Schaar et al. 1997). If microinjection of CENP-A-specific antibodies can prevent CENP-A from assembling onto centromere/kinetochore structures, it would then be possible to examine the putative function of CENP-A during centromere assembly and mitosis. We have used the direct needle microinjection method to monitor the effects of the antibody on the progress of individual cells during interphase and mitosis.

In a first series of experiments, HeLa cells synchronized at the G1/S boundary by a double thymidine block were released, microinjected at various times afterward and then left with the anti-CENP-A for different periods of time before being fixed for analysis by immunofluorescence and phase contrast microscopy. As shown in Fig. 2, we found that cells injected relatively soon after thymidine release

Fig. 3a–l. Phenotypes of HeLa cells injected in mitosis with affinity-purified anti-CENP-A antibodies. Cells blocked with nocodazole were injected and immediately released and analyzed by immunofluorescence with fixation at 35 min (**a**–**c**), 1 h 20 min (**d**–**f**), 2 h 20 min (**g**–**i**) and 11 h 15 min (**j**–**l**). Cells were visualized by phase contrast (**a**, **d**, **g**, **j**) and DAPI staining for DNA (**b**, **e**, **h**, **k**). For centromere staining, injected cells were visualized by incubation with fluorescein-labeled secondary antibody. Cells in prometaphase (**c**), metaphase (**f**), cytokinesis (**i**) and after division (**l**) were identified. Microinjection of anti-CENP-A antibodies does not appear to block any mitotic stage in cells released from nocodazole synchronization. Original magnification 100× (**d**), 40× (**a**, **g**, **j**). *Bar* represents 10 μm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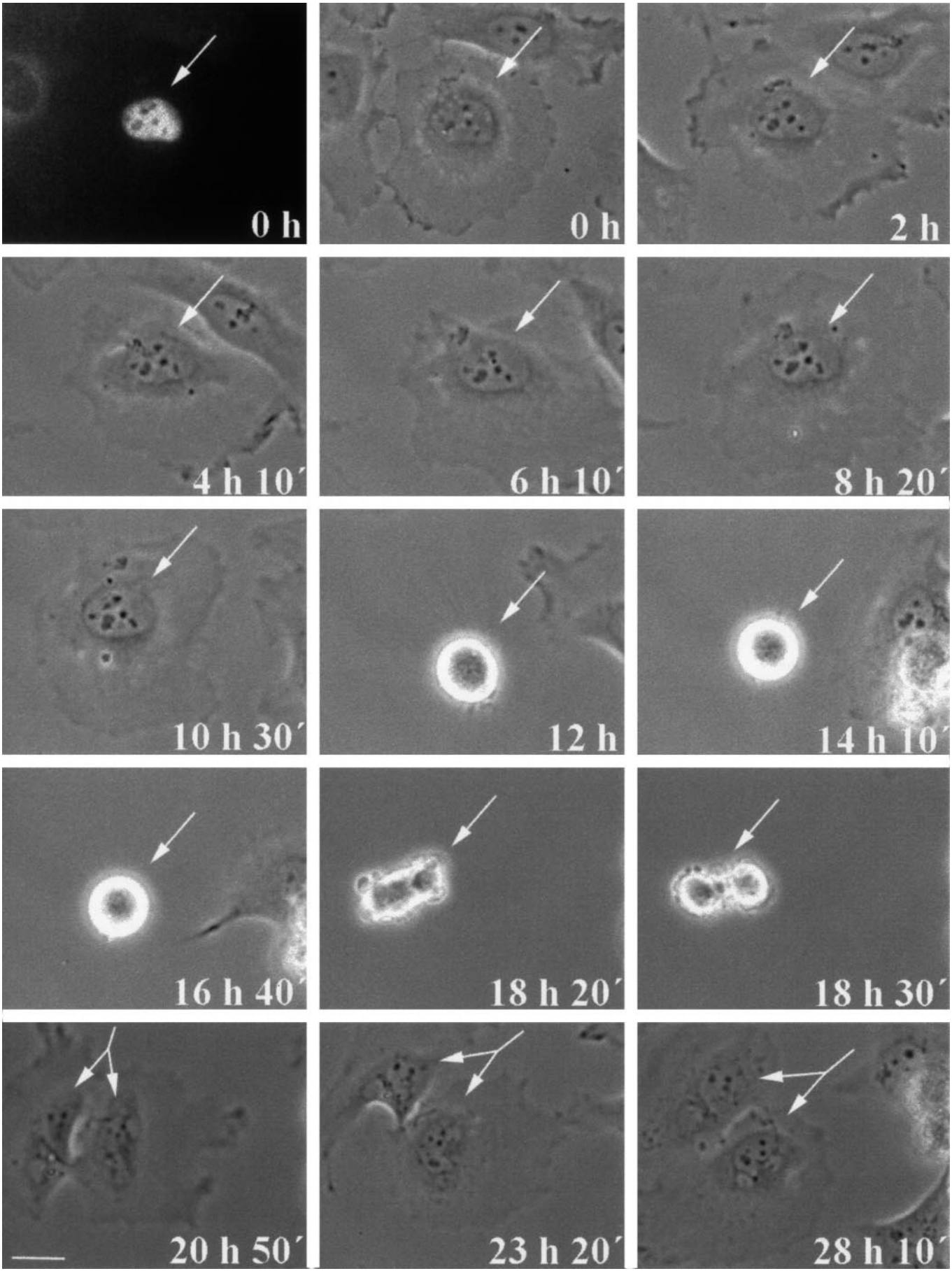
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(1–4 h), were blocked in interphase and showed a distinctive morphology. Visualized by phase contrast, the nuclei appeared small and very condensed, with a granular texture of the cytoplasm (Fig. 2g, j, m) and cells showed clear signs of loss of viability, as determined by their appearance and morphology. DNA staining of injected cells showed a high chromatin density, in comparison with cells not injected (Fig. 2h, n). Staining with fluorescein-labeled secondary antibody for CENP-A, despite initially producing some of the typical punctate pattern (Fig. 2f), in most cases indicated that CENP-A was associated in large stained clusters, probably as a result of atypical chromatin condensation (Fig. 2i, l, o). This effect was observed independently of the time between the injection of anti-CENP-A into the cells and processing for immunofluorescence (from 1.5–22 h) (Fig. 2i), but not initially when immunofluorescent staining was done only 30 min after anti-CENP-A injection (Fig. 2c, f). Furthermore, when microinjection was performed at 6–7 h or later after thymidine release, the interphase arrest caused by the anti-CENP-A injection was only observed eventually (arrowhead in Fig. 2p–r), and cells showed morphology and CENP-A staining quite similar to noninjected cells (Fig. 2p–r, s–u). In these cases, after cell division, CENP-A staining was still observed (Fig. 2r, u) although some cytoplasmic staining was also present (Fig. 2r). Microinjection of whole anti-CENP-A serum showed, in all the cases analyzed, a similar pattern to that observed with affinity-purified CENP-A antibody. This was performed as a control to ensure that the affinity chromatography step had not introduced any additional component responsible for the effect observed.

A series of microinjection experiments using various dilutions of anti-CENP-A indicated that the interphase arrest was similar at concentrations in the range of 1–5 mg/ml of affinity-purified anti-CENP-A. Controls cells injected with rabbit preimmune serum or PBS continued to grow normally, while cells injected with human autoimmune CREST serum were blocked in mitosis as described previously (Bernat et al. 1990) (data not shown). Based on the comprehensive controls used, we are confident that the interphase arrest observed in cells injected in early S-phase with anti-CENP-A is due only to the action of the antibody. From these experiments we concluded that some function of CENP-A can be blocked in interphase when anti-CENP-A is made available for binding to the antigen at a very early stage, immediately following mRNA synthesis (Shelby et al. 1997) (see Discussion).

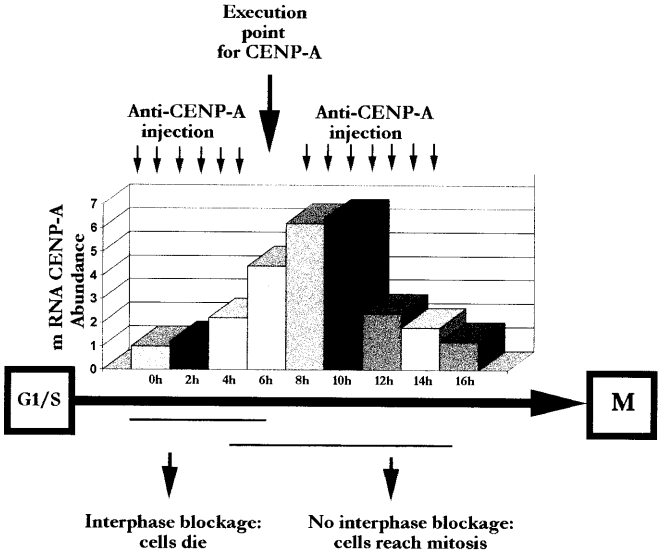

Fig. 5. Putative execution point for CENP-A in the cell cycle. Our observations of HeLa cells microinjected with monospecific affinity-purified anti-CENP-A antibodies lead us to suggest that, at an early point in interphase before maximum CENP-A mRNA accumulation (histogram bars from Shelby et al. 1997), anti-CENP-A executes a disruption of the CENP-A function for certain stages of centromere/kinetochore assembly during interphase. However, once this point is passed, the cells proceed to mitosis and divide

It might be expected that, since CENP-A is a centromere-associated antigen, anti-CENP-A would also disrupt some mitotic events. With that in mind, we examined nocodazole-blocked HeLa cells microinjected with anti-CENP-A. The motions of chromosomes in microinjected mitotic cells was analyzed by immunofluorescence at various times (see legend to Fig. 3). Examination of injected cells by using fluorescein-labeled secondary antibody showed that CENP-A was distributed at the centromeres at different stages of mitosis with a typical punctate staining (Fig. 3c, l). The microinjected cells seem to progress normally through mitosis to complete cell division after being exposed to anti-CENP-A for several hours (Fig. 3l). Our conclusion from these observations is that microinjection of anti-CENP-A antibodies into mitotic blocked cells does not prevent completion of mitosis by interfering with any mitotic check points, since these cells progressed normally to division. This result correlates well with that found by Bernat et al. (1990) using human polyclonal autoimmune anti-centromere serum containing some significant levels of anti-CENP-A among other anti-CENP immunoglobulins.

In order to study further the functional role of CENP-A during mitosis, we observed microinjected cells by time-lapse imaging microscopy. As controls, cells were studied without injection, and after injection with Texas Red solution (see Materials and methods) and with human CREST anticentromere serum. A representative example of monitored cells injected 4–5 h after G1/S is shown in Fig. 4. It was observed that the cells progressed through all the various stages of the cell cycle. It is noteworthy that a significant delay in the time taken to achieve mitosis was found. Compared with 1 h taken for normal cells (and

Fig. 4. Time-lapse imaging microscopy of a single HeLa cell microinjected with affinity-purified anti-CENP-A antibodies. The same cell was recorded in vivo by phase contrast microscopy. Cells synchronized by a double thymidine block were released for 4–5 h before microinjection. The panels show cell progression from the time of injection (0 h) of anti-CENP-A (5 mg/ml) through the various stages, including S- and G2-phase (0–10 h), mitosis (11–19 h approximately) and cell division. Although a clear effect of anti-CENP-A in delaying mitosis was observed, no evidence for mitotic arrest was identified. We have also monitored some cells arrested in interphase that do not reach mitosis after microinjection. These represented cells injected early in S-phase after the release of thymidine block, showing the same arrested stage in interphase as those indicated in Fig. 2 (data not shown). *Bar* represents 10 μm

cells injected with preimmune serum) to complete mitosis, anti-CENP-A-injected cells take up to 5–7 h to reach the next interphase stage. Further, cells injected with human anticentromere serum (with a high titer to CENP-B) showed a mitotic block, which corroborated the results of a previous study (Bernat et al. 1990).

Lastly, cells injected at the earliest stages of their S-phase were found to be blocked in S/G2 with a clear loss of viability, and did not proceed to mitosis (data not shown). These cells are very probably at a similar stage to those described in Fig. 2, which were blocked during interphase through interference with some execution point for CENP-A before mitosis (Fig. 5). These results suggest that CENP-A is required at some point for centromere/kinetochore assembly before chromosome condensation.

Discussion

CENP-A is a major autoantigen in CREST patients and is located at active centromeres (Choo 1997; Warburton et al. 1997). Its location, as indicated by light microscopy, at the inner kinetochore plate, is close to that of another centromeric antigen, CENP-C. The biochemical structure of CENP-A as a histone H3-like protein suggests that it plays an essential role in the centromeric chromatin organization (Palmer et al. 1987; Shelby et al. 1997). The well-conserved C-terminal region of CENP-A is indispensible for targeting the protein to the centromere (Sullivan et al. 1994). In contrast, the function of the more diverse N-terminal region is unclear, although it may play a structural role, such as interacting with other centromeric proteins for linking kinetochore subunits (Kalitsis et al. 1998).

Here we have studied the effect on cell cycle progression produced by microinjection of specific affinity-purified anti-CENP-A antibodies in HeLa cells. Injection of anti-CENP-A interferes with at least one interphase/G2 process with a execution point before mitosis (Fig. 5). This explains the accumulation of cells blocked at interphase when the injection of the antibody was performed from 1 to 4–5 h after releasing the G1/S boundary (Fig. 2). The morphology of arrested cells as visualized by phase contrast suggests a cellular stage of nonviability (Fig. 2n), which could be explained by an apoptotic mechanism. However this point will require further study. When microinjection of anti-CENP-A was performed 5–6 h and later after releasing the G1/S boundary, the effect was diminished and disappeared, with the cells reaching mitosis and progressing to the next interphase (Fig. 2p, u, and video images in Fig. 4). Apparently the required interphase events (putatively the assembly of specific centromeric nucleosomes containing CENP-A) sensitive to antibody inhibition have already been completed by the time the microinjection is performed later in S-phase.

Considering the staining pattern of CENP-A in normal cells during the cell cycle, and results from a previous report (Bernat et al. 1990), we initially expected to find some effect of anti-CENP-A antibodies in mitosis. To study that in more detail, we injected nocodazole-blocked HeLa cells and analyzed the subsequent mitotic steps after releasing the drug. Staining by immunofluorescence revealed that CENP-A was present at the centromeres of injected cells that were released from nocodazole block. Further, the cells progressed through to mitosis with anti-CENP-A bound to their centromeres (Fig. 3a–f) and eventually divided (Fig. 3g–l). This was demonstrated in more detail by time-lapse imaging microscopy of living injected cells (Fig. 4). Again, cells were found to reach mitosis and process to the next interphase. However, a delay of several hours (4–6 h) for the cell to pass mitosis was observed (Fig. 4). The initial interpretation of these results is that once CENP-A has organized the kinetochore (inner plate) of the centromeric chromatin in interphase the cell is protected from disruption by the monospecific anti-CENP-A antibody used and no adverse effect in mitosis was observed. This also suggests that CENP-A does not play a critical role during chromosome movement in metaphase or anaphase, as occurs with other CENPs (Tomkiel et al. 1994; Schaar et al. 1997). Further, the N-terminal epitope recognized by the CENP-A antibody used in this study, should be accessible for antibody interaction without disturbing other centromeric functions during mitosis, although it does cause some minor effects, as indicated by the mitotic delay observed. The reason for this delay is unknown but may be due to the presence of anti-CENP-A molecules bound to CENP-A at the kinetochore interfering with the machinery of motor proteins and microtubules during chromosome movement. Obviously, this result does not rule out the possibility of a specific role of CENP-A during mitosis that was not observed by the microinjection of the anti-N-terminal CENP-A antibody used in this study.

Our experiments reveal the existence of anti-CENP-A-disrupted events during interphase/G2 that are essential to the cell's viability in reaching mitosis. These events may include some important stages in the assembly of the active kinetochore structure necessary to attract replicated heterochromatin and associated proteins (CENP-B) for chromosome condensation in early prophase. Late S-phase is the time of replication of α-satellite DNA, which is a major component of the human centromere (Jabs and Persico 1987). CENP-A is thought to replace histone H3 in centromeric nucleosomes and its expression is uncoupled from H3 synthesis during S-phase, with CENP-A mRNA reaching a peak at G2 (Shelby et al. 1997). Further, CENP-A synthesis is coupled with centromere replication in mid to late S-phase (O'Keefe et al. 1992). In accordance with these observations, it could be predicted that CENP-A forms a nucleosomal structure required for mitotic events (Bernat et al. 1990; Shelby et al. 1997; Kalitsis et al. 1998). We now suggest that anti-CENP-A antibodies disrupt that assembly and prevent the formation of the inner plate essential for a correctly functioning kinetochore. This hypothesis also implies that progression to early prophase is disrupted by interference with centromere maturation and kinetochore assembly and activation before mitosis. The current availability of specific antibodies to other CENP antigens located at the kinetochore plates (CENP-C, CENP-E) will permit the analysis of this interference and the study of the time taken for the step-by-step arrangement of centromere/kinetochore assembly during maturation to mitosis.

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