Journal of Cell Science 111, 2551-2561 (1998)
Printed in Great Britain © The Company of Biologists Limited 1998
JCS7235

Expanding the role of HsEg5 within the mitotic and post-mitotic phases of the cell cycle

C. M. Whitehead[1,*] and J. B. Rattner[1,2,3,‡]

Departments of [1]Medical Biochemistry, [2]Anatomy and [3]Oncology, The University of Calgary, Calgary, Alberta, Canada
*Present address: Tumor Biology Group, Mayo Clinic Foundation, Rochester, Minnesota, 55905, USA
‡Author for correspondence (e-mail: rattner@acs.ucalgary.ca)

Accepted 2 July; published on WWW 13 August 1998

SUMMARY

The BimC family of kinesin like proteins are involved in spindle dynamics in a wide variety of organisms. The human member of this family, HsEg5, has been implicated in centrosome separation during prophase/prometaphase and in the organization of in vitro mitotic asters. HsEg5 displays a complex distribution during mitosis, associating with the centrosomes, spindle microtubules, specific regions of the intracellular bridge and a microtubule bundle that forms in association with the post-mitotic migration of the centrosome. In an effort to determine the function of HsEg5 during late mitotic events and refine its proposed function during early mitotic centrosome separation, we microinjected antibodies specific to HsEg5 into HeLa cells during various stages of mitosis. In the presence of HsEg5 antibodies we find that the microtubule arrays responsible for both pre- and post-mitotic centrosome movement never form. Similarly, the microtubule bundle within the intracellular bridge becomes prematurely altered following karyokinesis resulting in the loss of the microtubule array at either end of the bridge. In addition, some peri-centrosomal material at the spindle poles becomes fragmented and the distribution of the spindle protein NuMA becomes more concentrated at the minus ends of the spindle microtubules. Our study also provides direct evidence that there is a link between post-mitotic centrosome migration and Golgi complex positioning and reformation following mitosis. We conclude that HsEg5 plays a recurrent role in establishing and/or determining the stability of specific microtubule arrays that form during cell division and that this role may encompass the ability of HsEg5 to influence the distribution of other protein components associated with cell division

Key words: HsEg5, Centrosome, Golgi complex, Intracellular bridge

INTRODUCTION

Cell division requires a complex set of proteins, each of which can play either a single or multifaceted role within the division process. One protein that may be required for a variety of mitotic events is HsEg5, the human homologue of *Xenopus* Eg5 (Blangy et al., 1995; LeGueliec et al., 1991). HsEg5 is a member of the BimC family of kinesin related proteins whose members are characterized by a conserved, globular motor domain at the amino terminus followed by a non-conserved, rod-like helical coiled-coil domain and a BimC box at the carboxyl terminus (Endow, 1991; Hagan and Yanagida, 1992; Saunders et al., 1995). The phosphorylation state of the BimC box is correlated with spindle localization (Blangy et al., 1995; Sawin and Mitchison, 1995). It remains unclear whether this phosphorylation event is required for its multimerization into an active complex or for direct binding to microtubules (Walczak and Mitchison, 1996).

Cytological studies have revealed that HsEg5 has a complex distribution in dividing cells. First detected at the centrosomes of G_2/prophase cells, it is subsequently found along the microtubules associated with separating centrosomes and those forming the spindle, including kinetochore microtubules. At late anaphase the protein is detected at the spindle equator where it persists during telophase and is incorporated into the intercellular bridge (Rattner et al., 1992; Whitehead et al., 1996b). Initially when stained for HsEg5 the intracellular bridge shows four distinct zones of reactivity: one at either side of the midbody and one at each end of the bridge. The amount of HsEg5 found within the bridge declines as the bridge matures. Following karyokinesis but before the completion of cytokinesis, HsEg5 is found in association with the microtubule bundle that forms in concert with post-mitotic centrosome repositioning in each daughter cell (Mack and Rattner, 1993; Rattner et al., 1992; Whitehead et al., 1996b).

To date, HsEg5 function has been restricted to two early mitotic events. First, in vitro data, using a cell free HeLa mitotic extract, indicates that HsEg5 is required for the organization of mitotic asters and that it functions in concert with NuMA, dynein and dynactin (Gaglio et al., 1997b). It has been suggested that the individual forces produced by each of these proteins must be balanced by the forces produced by the other proteins of the aster in order to form a morphologically correct mitotic aster (Gaglio et al., 1997b). The complex

relationship between these spindle proteins is supported by the recent report that the tail of HsEg5 can interact with the p150 subunit of the dynactin complex and that this interaction is enhanced when HsEg5 is phosphorylated (Blangy et al., 1997). In addition, the transfection of various HsEg5 mutants into HeLa cells resulted in a 'rigor' phenotype associated with increased microtubule bundling (Blangy et al., 1998). Secondly, the *Xenopus* homologue, Eg5, has been implicated in bipolar spindle formation by antibody depletion experiments utilizing *Xenopus* egg extracts (Sawin et al., 1992). In vivo data, produced by microinjection experiments using HeLa or Monkey CV-1 cells and antibodies to either Eg5 or HsEg5, indicated that HsEg5 is essential for centrosome separation at the onset of spindle formation (Blangy et al., 1995; Gaglio et al., 1997b). These studies failed to reveal any additional roles for HsEg5 during other phases of cell division.

Exactly how and why HsEg5 is involved in centrosome separation is unclear, and functional studies must take into account the variability inherent in this process. Centrosome separation at the onset of cell division can occur either prior to, or following nuclear envelope breakdown (NEB). If centrosomes complete separation prior to NEB, the associated microtubule array between them disassociates upon the completion of their movement. Subsequently, a second array forms at NEB that is incorporated into the mitotic spindle. In contrast, if centrosome separation is postponed to the period following NEB the microtubule array that forms in association with separation is incorporated into the forming spindle. HsEg5 has been found in association with all these microtubule arrays (Blangy et al., 1995; Whitehead et al., 1996a,b).

One model of HsEg5 function suggests that it may play a role in mediating the interactions of antiparallel microtubules formed between opposing centrosomes (Blangy et al., 1995). However, this model does not account for the variability seen in centrosome separation. In some instances the degree of overlap between microtubules originating from opposing centrosomes during movement can either be extensive or minimal, suggesting that the motive force originates outside the region of overlap (Waters et al., 1993). Also, prophase centrosomes can move independently (with no overlapping antiparallel microtubule arrays) in cells treated with cytochalasin D (Whitehead et al., 1996b). Independent centrosome movement is also found in untreated mitotic cells following the formation of the cleavage furrow but prior to the completion of cytokinesis. During this period the centrosome of each daughter cell migrates from its original polar position, around the reforming nucleus, to a site adjacent to the intracellular bridge (Mack and Rattner, 1993). HsEg5 is found throughout the comet-like, microtubule bundle that forms in association with this movement and the direction of movement is dependent on an interaction between the actin cytoskeleton and the centrosome (Whitehead et al., 1996b).

The distribution of HsEg5 throughout mitosis and the post-mitotic period suggests that its role may extend beyond early mitotic events. Furthermore, the specific role of HsEg5 in prophase centrosome separation and spindle pole organization requires further definition. To address these points we raised antibodies to the non-conserved coiled-coil stalk domain of HsEg5 and used them in microinjection experiments on HeLa cells in different stages of cell division. Our results indicate that HsEg5 is essential for several sequential mitotic events and the common effect of inhibiting HsEg5 is to destabilize or prevent the formation of specific types of microtubule arrays. These arrays include those responsible for centrosome separation or relocation as well as those within the intracellular bridge. In addition our study indicates that HsEg5 is involved in the correct distribution of some mitotic proteins and this finding may underlie its function within specific microtubule arrays. Lastly, our study illustrates that inhibition of HsEg5 function following karyokinesis but preceding cytokinesis has important ramifications in the reorganization of the cytosol and its components, specifically the centrosome and Golgi apparatus.

MATERIALS AND METHODS

Cell culture and microinjection

HeLa cells (American Type Tissue Collection, Rockville MD) were maintained in Joklick's MEM medium with 10% bovine fetal calf serum and plated on acid-etched glass coverslips 24 hours prior to use. In some experiments HeLa cells were grown in the presence of cytochalasin D (Sigma, St Louis, MO) 0.3-0.9 μg/ml for 1-1½ hours prior to fixation or nocodazole (Sigma) 2 μg/ml for 12 hours. Cells were microinjected following a previously described procedure (Feramisco, 1979). Microinjections were performed at various stages of the cell cycle using one of two solutions. For control experiments a non-specific sheep IgG (Sigma) suspended in sterile Dulbecco's phosphate buffered saline (D-PBS) 2 μg/μl was used. The anti-HsEg5 solution contained an affinity purified, polyclonal anti-HsEg5 antibody (M4F) raised to the non-conserved coiled coil domain of HsEg5 (Whitehead et al., 1996a) at 1.5 μg/μl and non-specific sheep IgG at 2 μg/μl in D-PBS.

Indirect immunofluorescence

Microinjected cells were incubated for the desired amount of time and fixed for 10 minutes in ice-cold 100% methanol. The slides were air dried and if not used immediately, stored at −20°C until use. Fixed preparations were rehydrated in D-PBS and incubated for ½ hour at 37°C with the appropriate serum. Cy3-conjugated donkey anti-sheep (Jackson Labs, Mississauga, ON) was used to identify injected cells. Centrosomes were detected by the used of a previously characterized human autoimmune serum (Mack et al., 1998). NuMA was detected by the use of the mouse monoclonal antibody 1F1 (Compton et al., 1991) a generous gift from Dr Duane Compton. Gamma tubulin was detected with a monoclonal antibody (Accurate Chemical and Scientific Corp., Westbury, NY) The Golgi apparatus was detected by a human autoimmune serum which specifically recognized Golgin-97 (Griffith et al., 1997) a generous gift from Dr Marvin Fritzler. HsEg5 was detected by the use of a mouse polyclonal antibody (M5F) raised to a region distinct from that used to generate the HsEg5 antibody used for microinjections (Whitehead et al., 1996a). All primary antibodies were recognized with appropriate FITC- or Cy3-conjugated secondary antibodies (Jackson Labs). Tubulin was detected with a FITC-conjugated mouse monoclonal antibody, TUB 2.1, which recognized β-tubulin (Sigma). Samples were washed for 10 minutes in D-PBS between each antibody incubation. Specimens were counterstained with DAPI (4′,6-diamindino-2-phenyl-indole), mounted in 90% glycerol containing paraphenylenediamine and observed using a Zeiss Axiophot fluorescence microscope. Images were recorded on Ilford HP-5 film.

RESULTS

HsEg5 is essential for centrosome separation and centrosome organization

To assess the function of HsEg5, several previous studies have used antibodies raised to various domains of HsEg5 or Eg5 in

microinjection experiments utilizing either interphase or metaphase cells (Blangy et al., 1995; Gaglio et al., 1997b). These studies report that cells injected during interphase become arrested at prometaphase. Blocked cells contained a monopolar spindle indicating a failure in centrosome separation. To verify this result, we injected the cytoplasm of interphase HeLa cells with our antibody raised to the non-conserved coiled-coil stalk region of HsEg5 and allowed the passage of one cell cycle (24 hours) before fixation. In such preparations, 54/61 (88%) of injected cells progressed to mitosis and became blocked in prometaphase and contained a monopolar spindle (Fig. 1A-C). The remaining 7 cells were still in interphase. The detection of blocked cells 24 hours following injection indicates that the injected antibodies were stable throughout this time and able to effect HsEg5 function throughout the experimental period. In contrast, 48/50 (96%) of the control cells injected with non-specific sheep IgG progressed through mitosis with no detectable abnormalities as indicated by the formation of two daughter cells (Fig. 1D-F). The remaining 2 cells had not progressed to mitosis and remained in interphase. When the control cells were reacted with a previously characterized human autoimmune serum shown to react with a family of centrosomal proteins including pericentrin, ninein and Cep250 (Mack et al., 1998), each centrosome appeared as a well defined focus of reactive material (Fig. 1D). However, when this centrosomal antibody was used on blocked cells following injection with HsEg5 antibodies, a single open ring composed of multiple punctate foci was observed (Fig. 1A). This arrangement was not reported in the original microinjection study that used antibodies to HsEg5 (Blangy et al., 1995), but is similar to the distribution of NuMA found in cells injected with antibodies raised to Eg5 (Gaglio et al., 1997b). Interphase cells injected with anti-HsEg5 antibodies did not display the open-ring morphology until they reached prometaphase (data not shown) To determine the extent to which the distribution of HsEg5 was disrupted in our injected cells, blocked cells were stained with a second HsEg5 antibody (M5F) specific to a separate and distinct epitope in the tail region (Whitehead et al., 1996a). We did not observe HsEg5 staining in injected cells indicating an absence of HsEg5 within the mitotic apparatus (data not shown). Our data demonstrate that our antibodies to the stalk region of HsEg5 replicate the results of previous studies, are able to inhibit centrosome separation in HeLa cells, and indicate that HsEg5 is required for the proper distribution of centrosomal material in early mitotic cells.

HsEg5 is essential for centrosome separation but not initial spindle formation

As noted in the introduction, there is some flexibility in the timing and process of centrosome separation and spindle formation. This means that the centrosomes can exist in a number of configurations in prophase/prometaphase cells. In prophase cells, centrosomes can be in one of three configurations: unseparated; in the process of separation; or separated and not associated with microtubules (Waters et al., 1993; Whitehead et al., 1996b). In logarithmically growing HeLa cultures used in this study, the ratio of these morphologies is 2:1:1 (n=105, 50:30:25), respectively. In prometaphase cells, the centrosomes can either be unseparated and associated with spindle microtubules in a monopolar configuration, or they can be separating in concert with spindle formation. The ratio of these morphologies in our logarithmically growing HeLa cultures is approximately equal (n=100 52:48). These various pre-mitotic centrosome configurations are summarized in Fig. 2. Based on our microinjection results using interphase cells, injection of antibodies to HsEg5 into prophase cells with unseparated centrosomes and prometaphase cells with unseparated centrosomes with monopolar spindles should produce cells blocked in prometaphase. To verify this prediction and to ascertain the effect of the HsEg5 antibody on the other

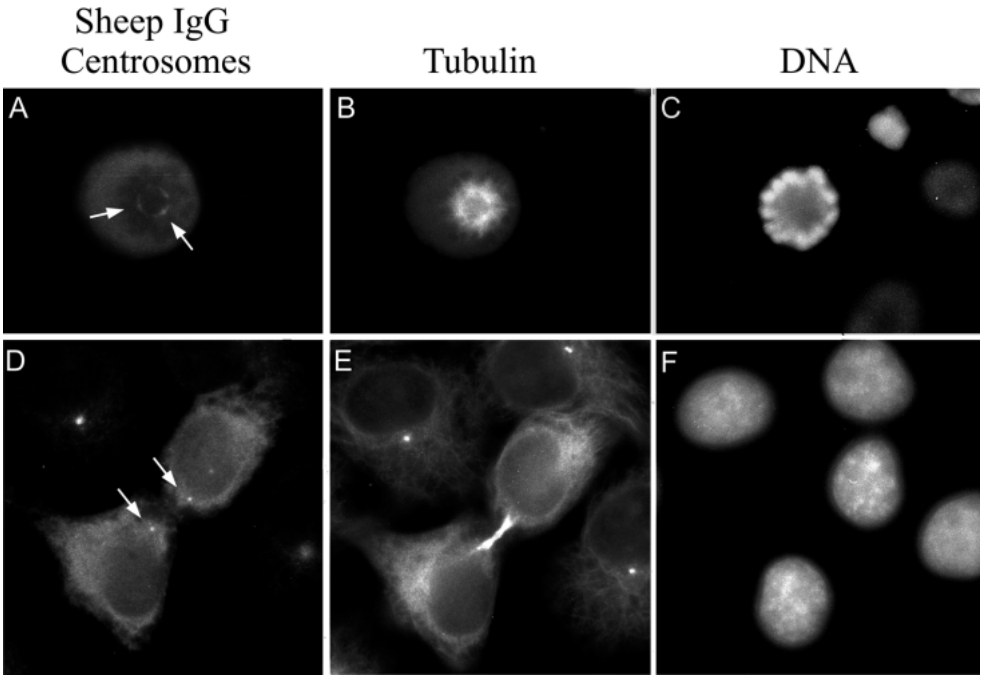

Fig. 1. A comparison of interphase cells injected with non-specific sheep IgG/anti-HsEg5 antibodies (A-C) or non-specific sheep IgG alone (D-F) and stained for sheep IgG (A,D), centrosomal material (A,D), tubulin (B,E) and DNA (C,F). The control injected cells (D-F) progressed through mitosis and the centrosomes are found in the correct post-mitotic position adjacent to the intracellular bridge (see arrows, D). Cells injected with anti-HsEg5 antibodies (A-C) become blocked in prometaphase with a ring-like, fragmented pole (see arrows, A). ×1,000.

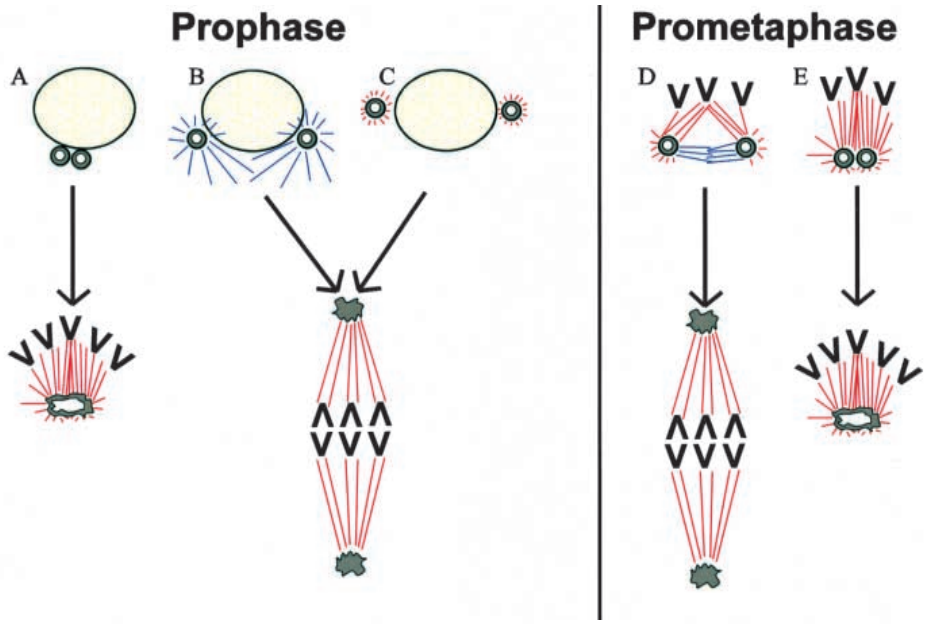

Fig. 2. The position of the centrosomes, in prophase and prometaphase cells, at the time of microinjection of anti-HsEg5 antibodies determines outcome. In prophase cells centrosomes can exist in one of three configurations: (A) unseparated, (B) separating, (C) separated, while prometaphase centrosomes can exist in one of two configurations: (D) separating or (E) unseparated. These configurations, after injection of anti-HsEg5 antibodies, led to either a monopolar spindle with a ring-like pole or a bipolar spindle with diffuse spindle poles. Microtubule arrays associated with centrosome separation are blue and spindle microtubules are red. The centrosomal/spindle pole material is grey.

centrosome configurations, we injected populations of both prophase and prometaphase cells. Prophase cells were identified by the rounded morphology of their cell body and the appearance of an enlarged but intact nucleus. Prometaphase cells were identified by the rounded morphology of their cell body and the appearance of dispersed chromosomes rather than the presence of a well-organised metaphase plate. Control injected cells progressed through mitosis with no observable abnormalities (Fig. 3A-C). When prophase HeLa cells were injected with antibodies to HsEg5 and then fixed 90 minutes later, two distinct configurations were observed. In the first configuration, cells progressed to form a typical metaphase plate and then proceeded through mitosis (Fig. 3D, injected cell on left). In the second, cells were blocked in prometaphase as predicted for cells injected prior to the initiation of centrosome separation (Fig. 3D, injected cell on right). The ratio of these two configurations in our prophase anti-HsEg5 injected HeLa cells was roughly equal (n=40, 20:20). Since the expected blocked population was 50% (i.e. the percentage of cells having unseparated centrosomes in our population at the time of injection), the remaining 50% of cells progressing through mitosis most likely represent the combination of cell populations with centrosomes in the process of separation plus those with centrosomes that had completed separation.

When prometaphase cells were microinjected, both prometaphase-blocked cells and cells capable of progressing through mitosis were observed, identical to configurations illustrated in Fig. 3D-F. The ratio of these two configurations was also roughly equal (n=45, 23:22). Again, this ratio correlates with the ratio of prometaphase cells showing centrosome separation (50%) compared to those possessing a monopolar spindle with unseparated centrosomes (50%). These results indicate that the absence of HsEg5 is catastrophic for microtubule arrays associated with centrosome separation but not for the second array that forms the mitotic spindle.

One draw back of the experiments described above is that we do not know with certainty the state of the centrosomes at the time of injection nor the relationship between the time of antibody availability on the efficiency of producing a block. Therefore to verify our assertion that the prometaphase blocked population arose from cells with unseparated centrosomes at the time of injection and assay the effect of a short exposure to anti-HsEg5 antibodies we prepared a homogeneous population of prometaphase HeLa cells using nocodazole. After a 12 hour nocodazole treatment the cells were released from the block and immediately injected with either anti-HsEg5 antibodies or non-specific sheep IgG. After 90 minutes the cells were fixed and stained to determine the position of their centrosomes. In the anti-HsEg5 injected cells all the centrosomes remained unseparated (n=32) while all the centrosomes in the non-specific sheep IgG injected cells proceeded to separate (n=35) (data not shown). This illustrates that mitotic injections are as effective as interphase injections in inhibiting HsEg5 function and verifies our assertion that the population of cells that had unseparated centrosomes after HsEg5 injection originate from cells that had unseparated centrosome at the time of injection. These findings are summarized in Fig. 2.

HsEg5 is essential for integrity of the centrosomes and the intracellular bridge during mitosis

Several structural abnormalities were detected in injected prophase or prometaphase cells that progressed through cell division. We observed that in some, but not all (15/20, 75%) cells with a bipolar spindle, the centrosome appeared more diffuse when stained with our human autoimmune sera that recognizes ninein, pericentrin and cep250 (compare arrows Fig. 3A,D). However, when these cells were stained with a monoclonal antibody to gamma-tubulin no apparent abnormal staining pattern was observed (data not shown). Thus HsEg5 appears to induce a rearrangement of some, but not all, components of the centrosome. The distribution of the spindle associated protein NuMA was also altered. Antibody staining revealed that in anti-HsEg5 injected cells NuMA loses its ‘V’ shaped spindle distribution and becomes concentrated at the minus ends of the spindle microtubules (Fig. 4D-F). Thus,

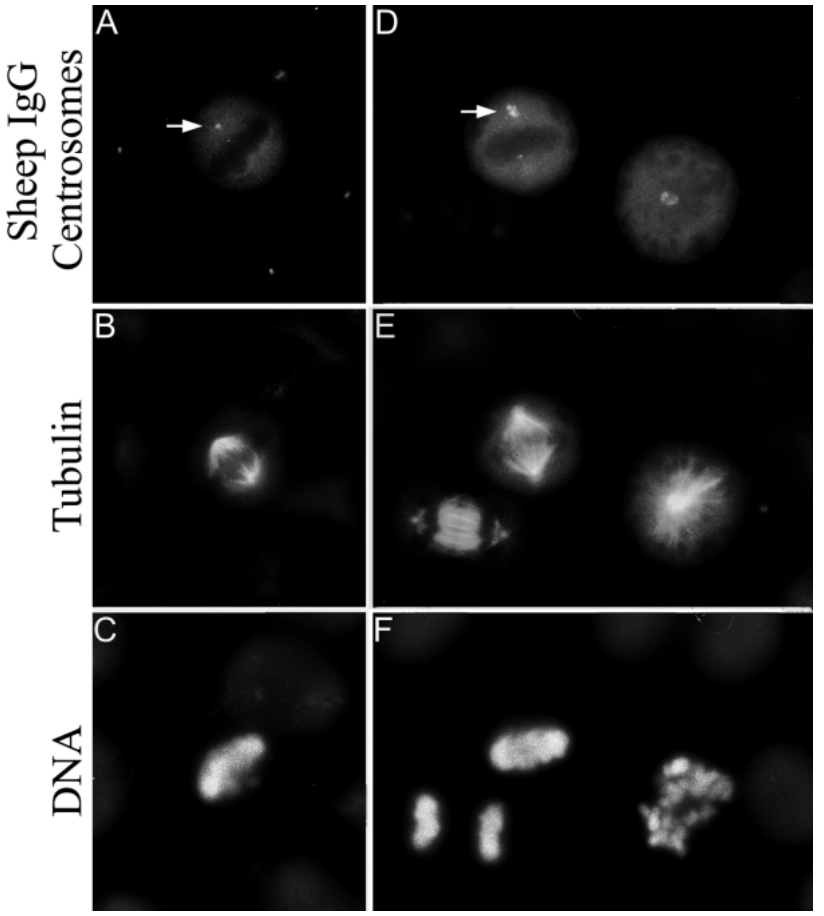

Fig. 3. A comparison of prophase/prometaphase cells injected with non-specific sheep IgG alone (A-C) or non-specific sheep IgG/anti-HsEg5 antibodies (D-F) and stained for sheep IgG (A,D), centrosomal material (A,D), tubulin (B,E) and DNA (C,F). In the cells injected with anti-HsEg5 antibodies the centrosomal material at the pole appears fragmented (compare arrows A,D). The second centrosome (D) in the injected cell on the left is out of the focal plane. Two distinct populations of cells injected with anti-HsEg5 were observed, one blocked in prometaphase (D, injected cell on right) and the other able of forming a bipolar spindle and progressing through mitosis (D, injected cell on left). ×1,000.

HsEg5 is essential for the correct distribution of several spindle pole components as well as spindle pole integrity, although this function is not essential for successful karyokinesis or mitotic progression.

A second abnormality observed in cells progressing through cell division after prophase/prometaphase injection was the formation of intracellular bridges with altered microtubule arrays. This abnormality was also observed in cells injected at metaphase and then fixed two hours later. Typically, in uninjected HeLa cells, HsEg5 is not initially detected within the equatorial region of the spindle during early anaphase (Fig. 5A-C). However, during late anaphase and telophase HsEg5 accumulates at the spindle equator and localizes to four regions: one on either side of the midbody, and one at each microtubule array at the end of the intracellular bridge (Fig. 5D-H) (Rattner et al., 1992; Whitehead et al., 1996a). As the intracellular bridge matures, the distribution of bridge microtubules changes. Newly formed intracellular bridges in HeLa cells are long, and contain bundles of microtubules derived from the spindle that extend into the cytoplasm of each daughter cell with a distinctive splayed morphology (Fig. 5H). As the bridge matures, the microtubule bundles shorten and become confined within the bridge (Fig. 5J), eventually displaying a pinched morphology at their ends proximal to the cell border (Fig. 5L). These microtubule dynamics are correlated with a loss of HsEg5, first at the ends of the bridge and then in its interior (Fig. 5G,I,K).

In our initial injection studies the frequency of telophase cells with shortened and pinched-off microtubule bundles within their intracellular bridges appeared to increase from that seen in the controls (Fig. 6A-D). That is, there appeared to be an increase in the population of cells that exhibited a more mature intracellular bridge morphology (compare Fig. 5K-L and Fig. 6D). To quantify this finding cells were injected at metaphase with either non-specific sheep IgG (n=50) or anti-HsEg5 antibodies (n=50), allowed to progress to late telophase (fixed two hours post-injection), and then scored for intracellular bridge morphology. The large majority of our control cells 42/50 (82%) displayed an extensive and well-formed bridge (as in Fig. 5G,H) while the small minority 8/50 (16%) displayed the characteristics of an older bridge, i.e. a shorter microtubule bundle with pinched ends (as in Fig. 5I-L). In cells injected with anti-HsEg5 antibodies, only 46% (23/50) displayed extensive well-formed bridges while 54% (27/50) displayed the characteristics of older bridges, suggesting that there was a more rapid morphological maturation of the bridge in anti-HsEg5 injected cells. When injected cells were fixed 6-12 hours post-injection, most cells (93%) had completed cytokinesis and formed two independent cells (data not shown). Thus, while HsEg5 is not essential for forming the intracellular bridge microtubule arrays it is required for the maintenance of its early, more extended morphology. Thus as in prophase, HsEg5 plays a specific role in determining the organization of a particular microtubule array although during telophase this role is subtle and spatially confined. It is interesting to note that the alterations induced

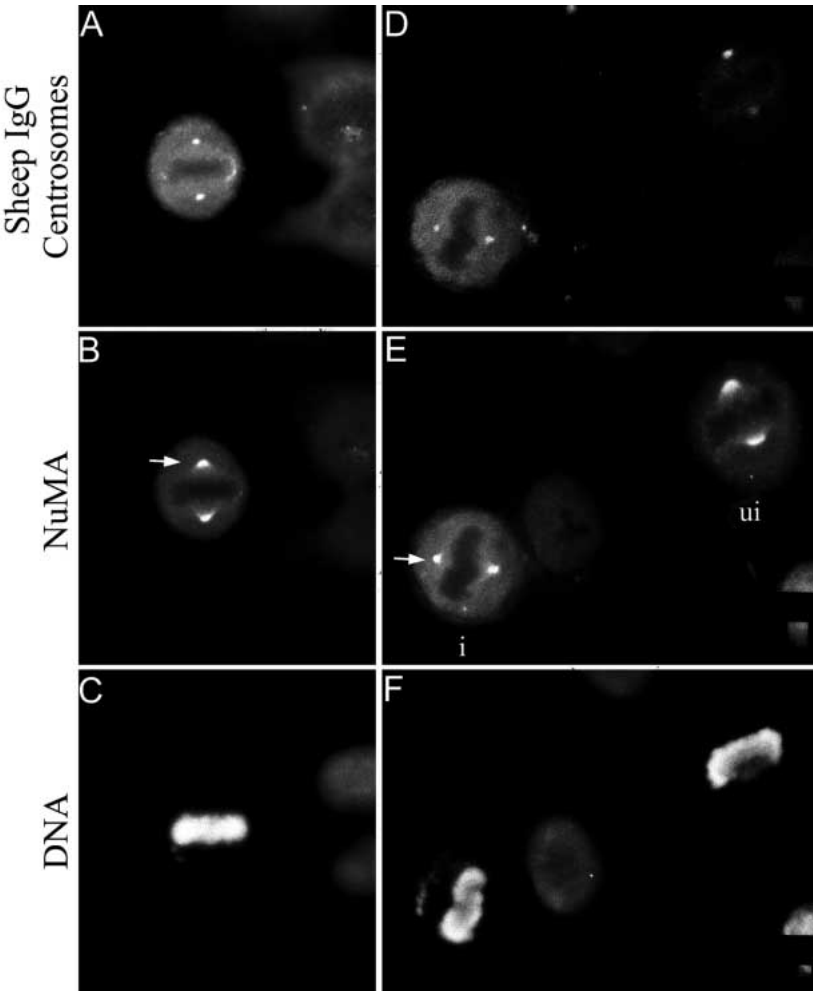

Fig. 4. A comparison of the distribution of NuMA in prophase/prometaphase cells injected with non-specific sheep IgG alone (A-C) or non-specific sheep IgG/anti-HsEg5 antibodies (D-F) and stained for sheep IgG (A,D), centrosomal material (A,D), tubulin (B,E) and DNA (C,F). NuMA loses its 'V' shaped spindle distribution and concentrates at the minus end of the spindle microtubules (compare arrows, B and E and the uninjected (ui) and injected (i) cells in E).

by the absence of HsEg5 during this period does not have a catastrophic effect on the completion of cytokinesis.

HsEg5 is essential for post mitotic centrosome migration

The process of post-mitotic centrosome movement that occurs prior to the completion of cytokinesis has been previously described, and involves the migration of the centrosome in each daughter cell from the site of the spindle pole to a point adjacent to the intracellular bridge (Fig. 7A-D) (Mack and Rattner, 1993). HsEg5 is present within the comet-like microtubule bundle that forms in association with this centrosomal movement (Whitehead et al., 1996b). In order to determine whether HsEg5 is essential for post-mitotic centrosome migration, HeLa cells were injected at metaphase with either anti-HsEg5 antibodies or non-specific sheep IgG, fixed two hours later, and scored for the position of each centrosome. In our control group (n=100) 21% of the cells had both centrosomes adjacent to the intracellular bridge, 43% had one there, and 38% still had both centrosomes at the poles. However, the centrosomes in only 16% of anti-HsEg5 injected cells were near the intracellular bridge, 24% of these cells had one centrosome that had lost its polar location, and in 60% both centrosomes remained at the poles (n=100). The appearance of some polar displaced centrosomes is not unexpected since there may be some passive displacement as a result of other cytoplasmic events such as cytoskeletal and nuclear reformation. In our control cells the microtubule bundle responsible for centrosome migration was readily apparent (100%, n=50) as illustrated in Fig. 7A-B while in our anti-HsEg5 injected cells (n=52) no evidence of this specific microtubule bundle was observed (Fig. 7E-F). Thus, HsEg5 is essential for the formation of a stable post-mitotic centrosome associated microtubule array and as a consequence, the relocation of the centrosome to a position directly adjacent to the intracellular bridge (summarized in Fig. 8). It is important to note that while we did not see the formation of the distinct microtubule bundles associated with centrosome movement in our anti-HsEg5 injected cells, the appearance of normal populations of cytoskeletal microtubules were observed (Figs 6D, 7F, 10D). This function mirrors that seen pre-mitotically and at late telophase, namely, the inhibition of HsEg5 activity only disrupts the formation and function of specific microtubule arrays that are associated with centrosome movement.

HsEg5 is essential for post-mitotic Golgi positioning

It has been suggested that centrosome migration to the intracellular bridge ensures that daughter cells have cytoskeletons with equal and opposite polarity (Mack and

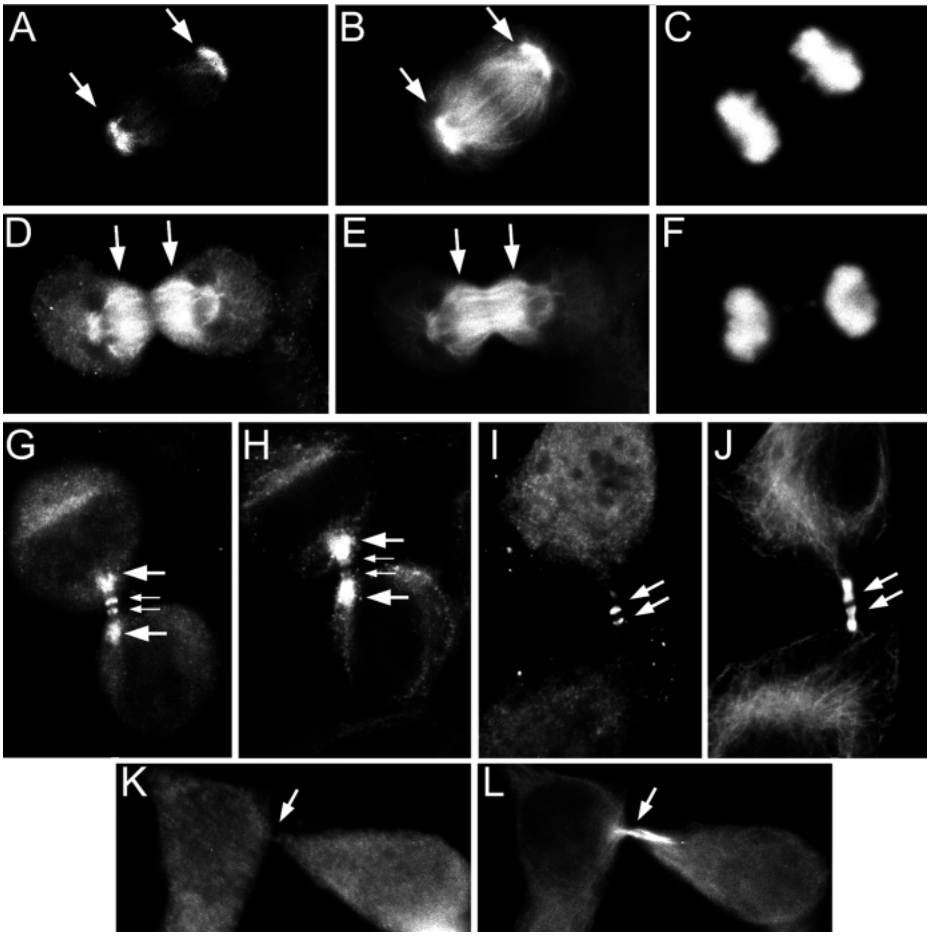

Fig. 5. The post-mitotic distribution of HsEg5 in uninjected HeLa cells stained for HsEg5 (A,D,G,I,K), tubulin (B,E,H,J,L) and DNA (C,F). During early anaphase HsEg5 is concentrated along kinetochore microtubules (arrows, A-C) and is not found along interpolar microtubules until late anaphase and the induction of furrowing (arrows, D-F). At telophase HsEg5 is associated with specific domains within the intracellular bridge, on either side of the midbody (small arrows) and along microtubules that extend into the cytoplasm (large arrows, G-H). As the intracellular bridge matures the amount of HsEg5 diminishes to two regions on either side of the midbody (arrows, I-J) and is eventually lost completely (arrows, K-L). ×1,000.

Rattner, 1993). If components associated with the interphase cytoskeleton depend on the correct position of the centrosome to determine their relative position in daughter cells following division, then their distribution should be affected in anti-HsEg5 injected cells where centrosome positioning is disrupted. To test this hypothesis we examined the distribution of the Golgi complex in cells injected at metaphase, with anti-HsEg5 antibodies. In HeLa cells the components of the Golgi

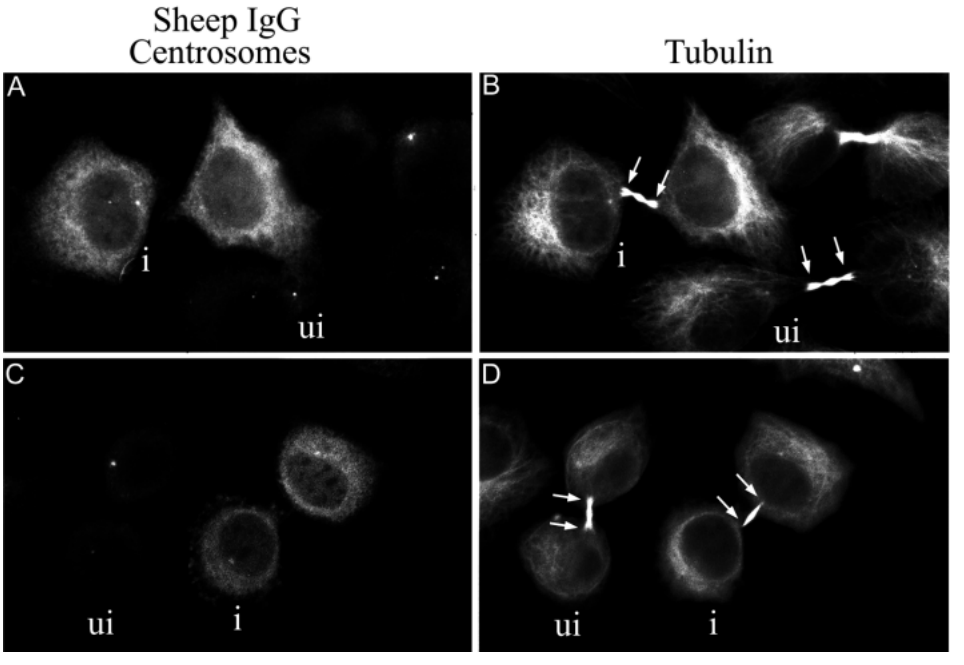

Fig. 6. A comparison of intracellular bridge morphology in cells injected at metaphase with non-specific sheep IgG alone (A,B) or non-specific sheep IgG/anti-HsEg5 antibodies (C,D) and stained for non-specific sheep IgG (A,C), centrosomal material (A,C) or tubulin (B,D). A population of intracellular bridges of control injected cells display a microtubule bundle with splayed ends that extend into the cytoplasm (arrows B, also compare injected (i) verses uninjected (ui)). In contrast, the intracellular bridges in cells injected with anti-HsEg5 antibodies exhibit a distinctive pinched morphology (arrows D, compare injected (i) verses uninjected (ui)). ×1,000.

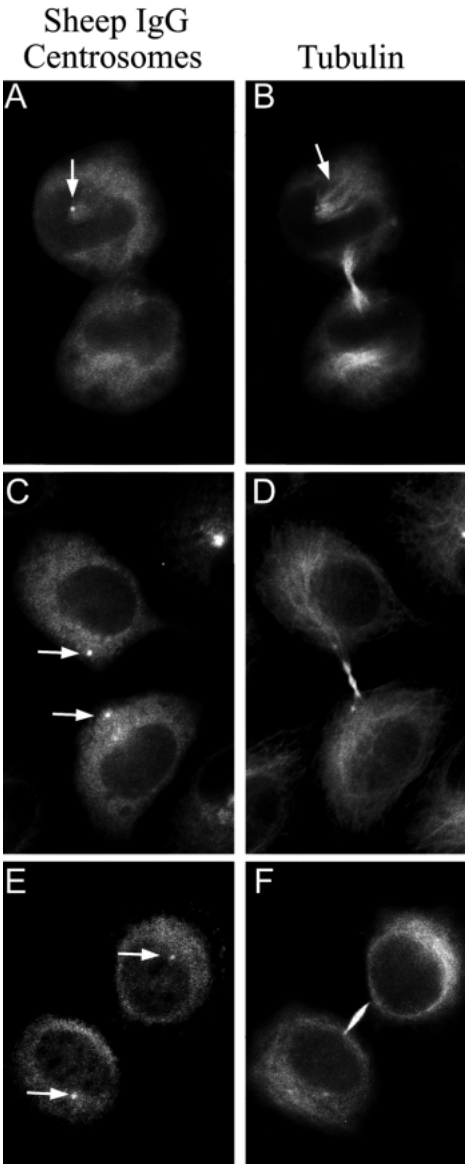

Fig. 7. A comparison of centrosome behaviour in post-mitotic cells injected at metaphase with non-specific sheep IgG alone (A-D) or non-specific sheep IgG/anti-HsEg5 antibodies (E-F) and stained for non-specific sheep IgG (A,C,E), centrosomal material (A,C,E) and tubulin (B,D,F). In control cells a microtubule bundle formed in association with the centrosome and all the microtubule profiles radiate in a comet-like fashion behind the centrosome (arrows, A-B) and the centrosomes were observed to migrate to a position adjacent to the intracellular bridge (arrows, C). In contrast, the centrosomes in cells injected with anti-HsEg5 antibodies fail to form a microtubule bundle directly associated with the centrosome and microtubule profiles are distributed throughout the cytoplasm. These centrosomes retained their polar location indicating a failure of migration (arrows, E-F). (E and F) A restricted view of Fig. 6C,D. ×1,000.

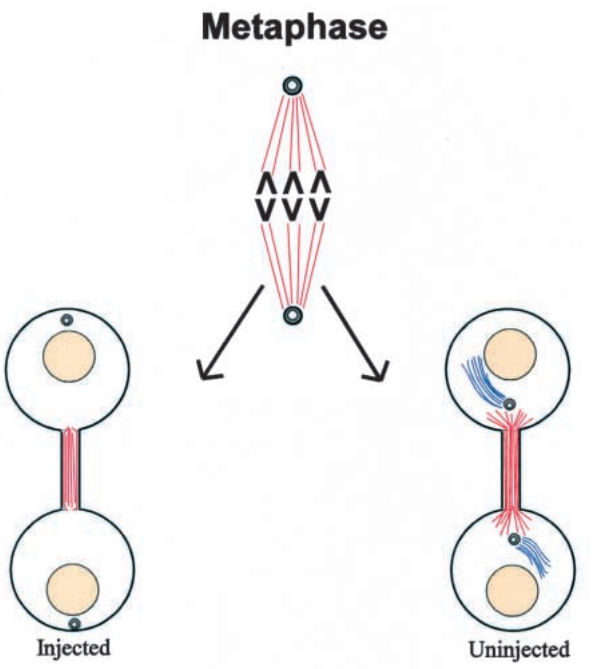

Fig. 8. Injection of anti-HsEg5 antibodies at metaphase blocked normal centrosome migration to the intracellular bridge and disrupted the morphology of intracellular bridge microtubules. The normal pattern in uninjected or control injected cells is shown on the right and the resulting morphology following injection with anti-HsEg5 antibodies, is shown on the left. Microtubule arrays associated with centrosome migration are blue and spindle microtubules are red.

complex undergo a stage-specific change in their distribution throughout mitosis that ensures a single peri-centrosomal Golgi complex in each daughter cell. The Golgi complex was observed using a human autoimmune serum that recognized Golgin-97 (Griffith et al., 1997). Golgin-97 is known to localize to the same Golgi components and exhibit a similar redistribution in brefeldin A treated cells as β-COP (Duden et al., 1991; Griffith et al., 1997; Orci et al., 1991). At metaphase, Golgi components are randomly distributed (Fig. 9A-B), but at the onset of anaphase they become localized to four distinct regions along each face of each chromosomal mass (Fig. 9C-D). At the completion of division, the two Golgi clusters seen on either side of anaphase chromosomes appear as a single cluster located between the reformed nucleus and the position of the intracellular bridge along with the centrosome (Fig. 9E-F). How the polar and the equatorial Golgi clusters come together is unknown.

While both post-mitotic centrosome and Golgi migration have been described (Mack and Rattner, 1993; Thyberg and Moskalewski, 1992), these two processes have never been linked together. However, if such a link exists, it may prove to be the vehicle for bringing the two Golgi populations together. To test whether Golgi movement is linked to centrosome migration, we grew HeLa cells in the presence of cytochalasin D to induce post-mitotic centrosomes to migrate along random and extended paths (Mack and Rattner, 1993; Whitehead et al., 1996b), and assayed for Golgi position relative to centrosome migration. Fig. 9G-H illustrates a treated cell in which a centrosome is migrating abnormally against the cell membrane causing it to evagenate. The resulting membrane evagination contains a centrosome at the distal most point and microtubules that extend back into the cell body (Mack and Rattner, 1993; Whitehead et al., 1996b). The Golgi can be seen to align along the microtubules and extend towards the centrosome, this arrangement was common in treated cells (95%, *n*=25) and

demonstrates the link between centrosome movement and Golgi placement. To confirm that this link also takes place in untreated HeLa cells, we studied the placement of the polar Golgi population with respect to the post-mitotic centrosome and its associated microtubule bundle. In each case Golgi material was found in association with the migrating centrosome (Fig. 9I-J). These observations confirm a relationship between centrosome migration and the post-mitotic relocation of Golgi components. Thus, post-mitotic centrosomal migration provides the vehicle for bringing together the two Golgi populations found in newly formed daughter cells.

These findings predict that in the absence of HsEg5, and consequently in the absence of centrosome migration, Golgi morphology should be perturbed. To test this hypothesis we injected metaphase cells with anti-HsEg5 antibodies and fixed 4½ hours post-injection. They were stained for Golgin-97 or tubulin, using a human autoimmune sera for Golgin-97 and a monoclonal antibody for tubulin (Griffith et al., 1997). The cytoskeletons of both control and anti-HsEg5 injected cells appeared morphologically normal (Fig. 10A-D). In contrast to control cells, the Golgi components in the vast majority (22/25, 88%) of injected cells failed to unify to a single focus and appeared randomly distributed within the cell (Fig. 10E-H).

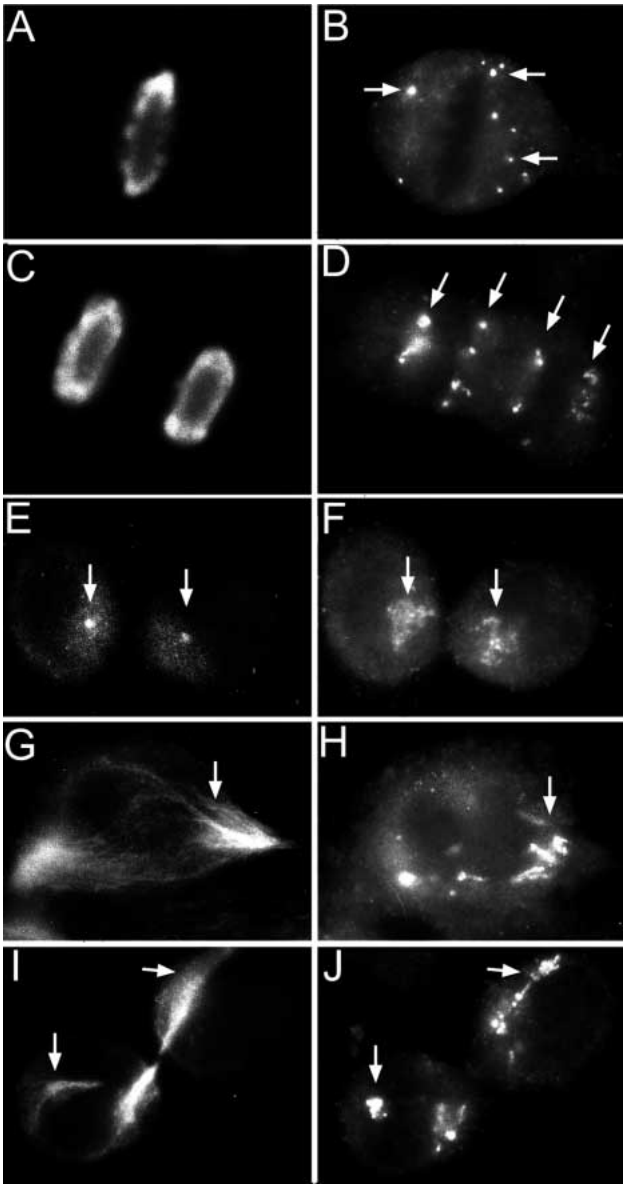

Fig. 9. The distribution of Golgi components through mitosis in HeLa cells stained for Golgin-97 (B,D,F,H,J), DNA (A,C), centrosomes (E) and tubulin (G,I). At metaphase, Golgi components are distributed randomly (arrows, A,B), while at anaphase, Golgi components become localized to both faces of each chromosomal mass (arrows, C,D). Subsequently the two groups in each daughter cell fuse and appear as a single unit proximal to the centrosome (arrows, E,F). A direct association of the Golgi with the microtubule bundles formed during post-mitotic centrosome migration was observed in both HeLa cells grown in the presence of cytochalasin D, which results in abnormal centrosome migration (arrows, G,H) or normal, untreated cells (arrows, I,J). ×1,000.

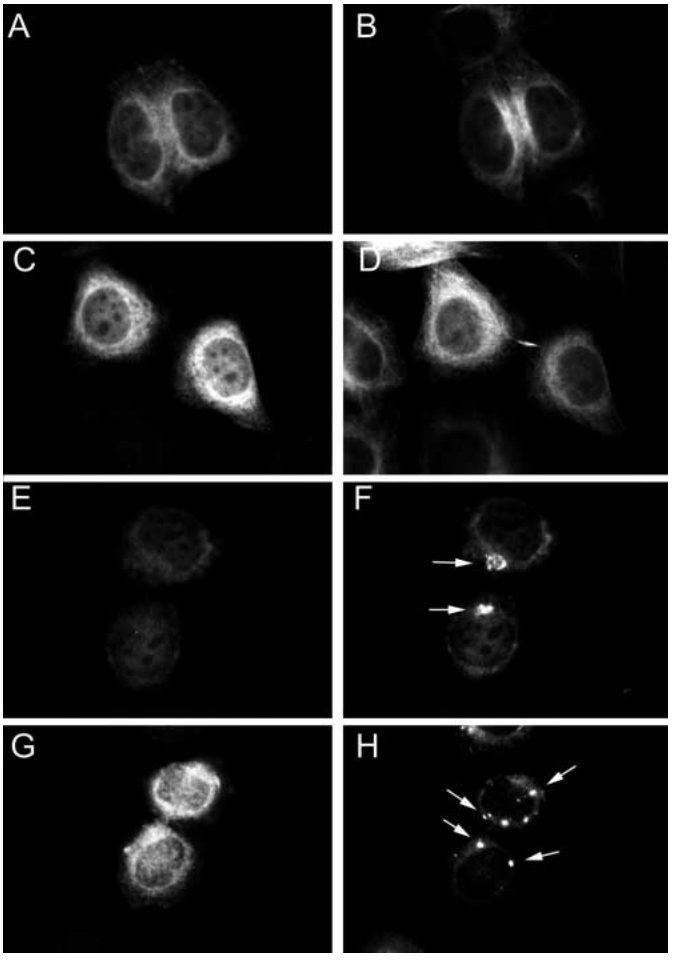

Fig. 10. A comparison of the distribution of the Golgi apparatus and the interphase tubulin cytoskeleton in HeLa cells injected at metaphase with non-specific sheep IgG only (A,B,E,F) or non-specific sheep IgG/anti-HsEg5 antibodies (C,D,G,H) and stained for non-specific sheep IgG (A,C,E,G), tubulin (B,D) or Golgin-97 (F,H). The interphase microtubule cytoskeleton appears normal in cells injected with non-specific sheep IgG (B) or anti-HsEg5 (D). The Golgi apparatus in control injected cells was observed in its normal location proximal to the former location of the intracellular bridge (arrows, F). In anti-HsEg5 injected cells the Golgi apparatus appeared fragmented throughout the cytoplasm (arrows, H).

Thus, the absence of HsEg5 not only results in a loss of post-mitotic centrosome migration but, as a consequence, interferes with the proper formation and positioning of the Golgi apparatus. At present this effect appears to be indirect because to date there has been no report of direct interactions between HsEg5 and any known Golgi protein. Nevertheless, our findings clearly indicate that proper post-mitotic HsEg5 function has ramifications on the process of cytoplasmic organization in each daughter cell.

DISCUSSION

The data presented in this report indicate that the plus-end directed kinesin-like motor protein, HsEg5 functions at multiple points from prophase through the early post-mitotic period. One recurring theme from our study is that the absence of HsEg5 either prevents or alters the formation and/or maintenance of some, but not all, microtubule arrays associated with cell division; specifically those associated with all forms of centrosome movement and the intracellular bridge. Further, we demonstrate that antibody binding to HsEg5 disrupts the normal distribution of some mitotic proteins suggesting that the function of HsEg5 within specific microtubule arrays may be related to its interaction with other mitotic components. Finally we demonstrate that the post-mitotic function of HsEg5 is essential to the proper organization of the centrosome and Golgi apparatus of each daughter cell.

Previous studies have indicated that HsEg5 is essential for centrosome separation at prophase. Our study extends and refines these observations and also shows that this protein is not only required for the movement of two centrosomes away from one another during early mitosis, but also for the post-mitotic movement of individual centrosomes. Our study underscores three important features of HsEg5. First, the finding that HsEg5 is essential for the movement of both single and paired centrosomes suggest that its function is inherent to each centrosome (Whitehead et al., 1996b). This is in agreement with the finding that centrosome movement is an inherent feature of each centrosome and its associated astral microtubules (Ault and Rieder, 1994; Waters et al., 1993). This conclusion, while not excluding models of HsEg5 function that require the presence of antiparallel microtubule arrays (Blangy et al., 1995), indicates that HsEg5 function can occur outside such arrays. Secondly, our study indicates that the requirement for HsEg5 is confined to specific types of microtubule arrays such as those associated with both pre- and post-mitotic centrosome movement. HsEg5 does not appear to be required for the establishment of pole to pole microtubule arrays formed after centrosome separation even though it is present in these arrays. Similarly, it is not essential for kinetochore microtubule maintenance. Thirdly, HsEg5 is essential for maintaining the proper relationship of a population of centrosomal proteins within the spindle pole. These in vivo findings are in agreement with in vitro studies of mitotic asters whose organization was disrupted by the immunodepletion of HsEg5 (Gaglio et al., 1997b). The monopolar spindle pole in prometaphase-blocked, anti-HsEg5 injected cells, fragments and exhibits a ring-like structure. Similarly, the centrosomal material in anti-HsEg5 injected cells that progressed to metaphase displayed a diffuse focal array (compare Fig. 3A,D). It is possible that the disruption of the relationship between HsEg5 and the distribution of specific mitotic proteins results in the instability or inhibition in the formation of specific mitotic microtubule arrays. While the absence of HsEg5 results in a change in protein distribution at the spindle poles during prometaphase/metaphase, this morphology was not seen in post-mitotic cells. It is possible that forces present in the spindle exaggerate the effect of HsEg5 removal, while in post-mitotic anti-HsEg5 injected cells, there is no microtubule array present to exert an external force on the centrosome.

One current model of spindle structure predicts that proper spindle pole morphology is maintained by a balance of forces produced by proteins (HsEg5, dynein, dynactin, NuMA) present in the spindle (Gaglio et al., 1997b). This model explains the change in the distribution of NuMA observed in our anti-HsEg5 injected cells. That is, NuMA appears to become concentrated at the minus ends of spindle microtubules as a consequence of the elimination of the plus-end directed force generated by HsEg5. In apparent conflict with this conclusion is the finding that in HsEg5-depleted in vitro asters, the distribution of NuMA becomes more diffuse. It is possible that this apparent discrepancy is due to the absence of some spindle components in the in vitro mitotic extracts or by the presence of additional forces found only in bipolar spindles in vivo. While it is apparent that a balance of the forces produced by HsEg5 and dynein is necessary for proper maintenance of the spindle pole, it is evident that these two proteins play distinctly different structural roles. The inhibition of dynein activity, specifically at metaphase, results in the separation of the centrosome from spindle microtubules (Gaglio et al., 1997a), a configuration not seen in our anti-HsEg5 injected cells.

The finding that HsEg5 is essential for the formation of specific microtubule arrays and the maintenance of correct protein distribution within the spindle pole is consistent with our observation that this protein is also required for the ordered maturation of the microtubule array within the intracellular bridge. In uninjected cells there is a reduction in the amount of HsEg5 present throughout the intracellular bridge as it matures. This is correlated with a shortening and pinching off of the microtubule bundle within the bridge. This maturation is enhanced by the injection of anti-HsEg5 antibodies into metaphase cells. Thus, HsEg5 appears to play a role in the stability of the minus end of the intracellular bridge microtubule array. It is tempting to speculate that the same types of force relationships that maintain spindle morphology may also act in the intracellular bridge during its morphogenesis. Despite the apparent requirement for HsEg5 in maintaining intracellular bridge morphology we were unable to detect any effect of this alteration on either cytokinesis or the organization of the actin cytoskeleton associated with the bridge (data not shown). Thus, HsEg5 is required for the maintenance of early intracellular bridge morphology but does not inhibit the establishment and final separation of daughter cells. It is possible that the altered bridge morphology would have an effect on centrosome migration since the bridge is used as a landmark in the orientation of this movement (Mack and Rattner, 1993). This particular feature could not be studied in this system because the injection of our anti-HsEg5 antibodies inhibited centrosome movement.

During mitosis the Golgi complex displays a unique distribution that ensures its equal partitioning into each daughter cell. At the onset of mitosis, the Golgi complex fragments into small groups of vesiculotubular elements

(Lucocq et al., 1987, 1989) that are randomly dispersed by metaphase but then coalesce into two distinct groups in each daughter cell during anaphase. The method by which these two groups come together and localize to a position between the reforming nucleus and the intracellular bridge upon the completion of mitosis has until now remained uncharacterised. Our study is the first to show a connection between post-mitotic centrosome repositioning and the unification of the two Golgi groups. The correct positioning of the Golgi complex is thought to facilitate the efficient secretion and distribution of proteins that are involved in the establishment of cell-matrix contacts and cell-cell communication channels (Moskalewski et al., 1994). It would be of interest to determine whether these functions are disrupted in anti-HsEg5 injected cells. During interphase, Golgi elements are known to associate with microtubules and use them to facilitate their localization to a position adjacent the centrosome (Ho et al., 1989). While additional motor proteins, dynein (Corthésy-Theulaz et al., 1992) and kinesin are known to associate with Golgi components, neither of these proteins appear capable of compensating for a loss of HsEg5 activity.

In conclusion, HsEg5 appears to play a similar function post-mitotically as it does earlier in cell division, that is, the organization and maintenance of specific microtubule arrays. These observations appear to correlate well with the report that HsEg5 mutants display an unregulated microtubule bundling activity (Blangy et al., 1998). The essential function of the HsEg5 dependent microtubule array during post-mitotic centrosomal migration not only establishes the polarity of daughter cells with respect to one another but participate in the organization and placement of cytoplasmic components. Thus, the centrosome is not only integrated into the mitotic spindle via HsEg5, but it also integrates it into the dynamic organization of the cell during early, post-mitotic, events.

The authors thank Drs Duane Compton and Marvin Fritzler for supplying us with antibodies to NuMA and Golgin-97, respectively, and Dr Brian Burke, Gary Mack and Olof Sandblom for helpful comments during the preparation of this manuscript. The work presented in this paper was supported by the Alberta Heritage Foundation for Medical Research (C.M.W.) and the National Cancer Institute with funds from the Canadian Cancer Society (J.B.R.).

REFERENCES

Ault, J. G. and Rieder, C. L. (1994). Centrosome and kinetochore movement during mitosis. *Curr. Opin. Cell Biol.* **6**, 41-49.

Blangy, A., Lane, H. A., Herin, P., Harper, M., Kress, M. and Nigg, E. A. (1995). Phosphorylation by $p34^{cdc2}$ regulates spindle association of human Eg5, a kinesin-related motor protein essential for bipolar spindle formation in vivo. *Cell* **83**, 1159-1169.

Blangy, A., Arnaud, L. and Nigg, E. A. (1997). Phosphorylation by $p34^{cdc2}$ protein kinase regulates binding of the kinesin-related motor HsEg5 to the dynactin subunit p150. *J. Biol. Chem.* **272**, 19418-19424.

Blangy, A., Chaussepied, P. and Nigg, E. A. (1998). Rigor-type mutation in the kinesin-related protein HsEg5 changes its subcellular localization and induces microtubule bundling. *Cell Motil. Cytoskel.* **40**, 174-182.

Compton, D. A., Yen, T. J. and Cleveland, D. W. (1991). Identification of novel centromere/kinetochore-associated proteins using monoclonal antibodies generated against human mitotic chromosome scaffolds. *J. Cell Biol.* **112**, 1083-1097.

Corthésy-Theulaz, I., Pauloin, A. and Pfeffer, R. (1992). Cytoplasmic dynein participates in the centrosomal localization of the Golgi complex. *J. Cell Biol.* **118**, 1333-1345.

Duden, R., Griffiths, G., Frank, R., Argos, P. and Kreis, T. E. (1991). β-COP, a 110 kd protein associated with non-clathrin-coated vesicles and the Golgi complex, shows homology to β-adaptin. *Cell* **64**, 649-665.

Endow, S. A. (1991). The emerging kinesin family of microtubule motor proteins. *Trends Biol. Sci.* **16**, 221-225.

Feramisco, J. R. (1979). Microinjection of fluorescently labeled alpha-actinin into living fibroblasts. *Proc. Nat. Acad. Sci. USA* **76**, 3967-3971.

Gaglio, T., Dionne, M. A. and Compton, D. A. (1997a). Mitotic spindle poles are organized by structural and motor proteins in addition to centrosomes. *J. Cell Biol.* **138**, 1055-1066.

Gaglio, T., Saredi, A., Bingham, J. B., Hasbani, M. J., Gill, S. R., Schroer, T. A. and Compton, D. A. (1997b). Opposing motor activities are required for the organization of the mammalian mitotic spindle pole. *J. Cell Biol.* **135**, 399-414.

Griffith, K. J., Chan, E. K., Lung, C., Hamel, J. C., Guo, X., Miyachi, K. and Fritzler, M. J. (1997). Molecular cloning of a novel 97-kd Golgi complex autoantigen associated with Sjögren's Syndrome. *Arthritis Rheum.* **40**, 1693-1702.

Hagan, I. and Yanagida, M. (1992). Kinesin-related cut7 protein associates with mitotic and meiotic spindles in fission yeast. *Nature* **356**, 74-76.

Ho, W. C., Allan, V. J., Meer, G., Berger, E. G. and Kreis, T. E. (1989). Reclustering of scattered Golgi elements occurs along microtubules. *Eur. J. Cell Biol.* **48**, 250-263.

LeGueliec, R., Paric, J., Couturier, A., Roghi, C. and Philippe, M. (1991). Cloning by differential screening of a *Xenopus* cDNA that encodes a kinesin-related protein. *Mol. Cell. Biol.* **11**, 3395-3398.

Lucocq, J. M., Pryde, J. G., Berger, E. G. and arren, G. (1987). A mitotic form of the Golgi apparatus in HeLa cells. *J. Cell Biol.* **104**, 865-874.

Lucocq, J. M., Berger, E. G. and Warren, G. (1989). Mitotic Golgi fragments in HeLa cells and their role in the reassembly pathway. *J. Cell Biol.* **109**, 463-474.

Mack, G. and Rattner, J. B. (1993). Centrosome repositioning immediately following karyokinesis and prior to cytokinesis. *Cell Motil. Cytoskel.* **26**, 239-247.

Mack, G. J., Rees, J., Sandblom, O., Balczon, R., Fritzler, M. J. and Rattner, J. B. (1998). Autoantibodies to a group of centrosomal proteins in human autoimmune sera reactive with the centrosome. *Arthritis Rheum.* **41**, 551-558.

Moskalewski, S., Popowicz, P. and Tyberg, J. (1994). Functions of the Golgi complex in cell division: formation of cell-matrix contact and cell-cell communication channels in the terminal phase of cytokinesis. *J. Submicrosc. Cytol. Pathol.* **26**, 9-20.

Orci, L., Tagaya, M., Amherdt, M., Perrelet, A., Donaldson, J. A., Lippincott-Schwartz, J., Klausner, R. D. and Rothman, J. E. (1991). Brefeldin A, a drug that blocks secretion, prevents the assembly of non-clathrin-coated buds on Golgi cisternae. *Cell* **64**, 1183-1195.

Rattner, J. B., Wang, T., Mack, G., Martin, L. and Fritzler, M. J. (1992). MSA-35: a protein identified by human autoantibodies that colocalizes with microtubules. *Biochem. Cell Biol.* **70**, 1115-1122.

Saunders, W. S., Koshland, D., Eshel, D., Gibbons, I. R. and Hoyt, M. A. (1995). *Saccharomyces cerevisiae* kinesin- and dynein-related proteins required for anaphase chromosome segregation. *J. Cell Biol.* **128**, 617-624.

Sawin, K. E., LeGuellec, K., Philippe, M. and Mitchison, T. J. (1992). Mitotic spindle organization by a plus-end-directed microtubule motor. *Nature* **359**, 540-543.

Sawin, K. E. and Mitchison, T. J. (1995). Mutations in the kinesin-like protein Eg5 disrupting localization to the mitotic spindle. *Proc. Nat. Acad. Sci. USA* **92**, 4289-4293.

Thyberg, J. and Moskalewski, S. (1992). Reorganization of the Golgi complex in association with mitosis: redistribution of mannosidase II to the endoplasmic reticulum and effects of brefeldin A. *J. Submicrosc. Cytol. Pathol.* **24**, 495-508.

Walczak, C. E. and Mitchison, T. J. (1996). Kinesin-related proteins at mitotic spindle poles: Function and regulation. *Cell* **85**, 943-946.

Waters, J. C., Cole, R. W. and Rieder, C. L. (1993). The force-producing mechanism for centrosome separation during spindle formation in vertebrates is intrinsic to each aster. *J. Cell Biol.* **122**, 361-372.

Whitehead, C. M., Winkfein, R. J., Fritzler, M. J. and Rattner, J. B. (1996a). The spindle kinesin-like protein HsEg5 is an autoantigen in systemic lupus erythematosus. *Arthritis Rheum.* **39**, 1635-1642.

Whitehead, C. M., Winkfein, R. J. and Rattner, J. B. (1996b). The relationship of HsEg5 and the actin cytoskeleton to centrosome separation. *Cell Motil. Cytoskel.* **35**, 298-308.