Phosphoproteins are components of mitotic microtubule organizing centers

(immunofluorescence microscopy/monoclonal antibody/centrosome/kinetochore/midbody)

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ABSTRACT Protein phosphorylation has been suggested as an important control mechanism for the events leading toward the initiation and completion of mitosis. Using a monoclonal antibody recognizing a class of phosphoproteins abundant in mitotic cells, we demonstrated the localization of a subset of these phosphoproteins to several discrete mitotic structures. Patchy immunofluorescence was present in the interphase nuclei, but a significant increase in nuclear immunofluorescence was apparent at prophase. Subsequent mitotic stages demonstrated that immunoreactive material was particularly apparent at microtubule organizing centers, namely, centrosomes, kinetochores, and midbodies. Intense centrosomal localization occurred at the prophase–prometaphase transition and persisted until the reformation of the nuclear membrane in early G₁. The cytoplasm of mitotic cells also contained immunoreactive material in sharp contrast to interphase cells that exhibited no cytoplasmic fluorescent staining. Much of the diffuse immunofluorescent cytoplasmic material was removed by a brief lysis of the cells with 0.15% Triton X-100 prior to fixation. The localization of the remaining immunoreactive material after detergent lysis to mitotic microtubule organizing centers suggests that they contain phosphoprotein structural components important, perhaps, in the mitotic phase–interphase transition.

The regulatory events controlling the initiation and completion of mitosis are poorly understood. It is apparent, however, that such varied cellular responses as reorganization of the microtubule network, chromosome condensation, centriole duplication, breakdown of the nuclear membrane, formation of the mitotic spindle, and cytokinesis must be coordinated closely for the cell to progress through mitosis.

One possible mechanism for controlling these mitotic events would be through protein phosphorylation and dephosphorylation. In several systems, increased levels of protein phosphorylation are cell-cycle dependent phenomena. During meiotic maturation of *Xenopus laevis* oocytes, the incorporation of ³²P into protein is maximal prior to mitosis (1). Similarly, in HeLa cells, nuclear matrix proteins are phosphorylated to a maximal extent during the premitotic (G₂) phase (2). More specifically, the three major protein components of the nuclear lamina, lamins A, B, and C, incorporate ³²P maximally at the time of nuclear-envelope dissolution (3). The presence of other mitosis-specific proteins has been detected also by two-dimensional gel electrophoretic analysis of synchronized HeLa cell populations (4).

Davis *et al.* (5) have reported on the properties of a monoclonal antibody that recognized a set of phosphorylated proteins abundant in mitotic cells. Using one of these monoclonal antibodies, MPM-2 (5), we report here the localization of immunoreactive material to the centrosome, the kineto-

chore, and the midbody of mitotic cells. These results support the concept that specific protein phosphorylation may coordinate the transition of cells from interphase to mitosis.

MATERIALS AND METHODS

Cell Culture. Rat kangaroo cells (Pt K1) were maintained in monolayer culture using Ham's F-10 medium supplemented with 10% fetal bovine serum. Cells were subcultured to glass coverslips 3–4 days prior to being used for immunofluorescence experiments. To increase the number of mitotic cells, the cultures were partially synchronized with a single 2.5 mM thymidine block for 24 hr. Cells were processed 14–16 hr after release from the block. Human foreskin fibroblasts were cultured in a similar manner with a 15% supplement of fetal bovine serum and were not exposed to thymidine.

Antibodies. A monoclonal antibody, MPM-2, reactive with phosphoproteins of HeLa mitotic cells was obtained and characterized as described (5). A monoclonal antibody raised against yeast tubulin was a gift of J. V. Kilmartin, and human anti-centrosomal serum was a gift from M. Kirschner.

Immunofluorescence Staining. Cells were briefly rinsed with phosphate-buffered saline (pH 7.4) (P_i/NaCl) and fixed in 0.7% glutaraldehyde/0.2% Nonidet P-40 in P_i/NaCl containing 0.002% sodium azide for 15 min. Coverslips were washed with P_i/NaCl and treated with NaBH₄ (1 mg/ml) for 15 min. After a second reduction with NaBH₄, coverslips were rinsed and incubated with the appropriate primary antibody for 60 min, rinsed again, incubated with fluorescein-conjugated goat anti-mouse immunoglobulin for 30 min, rinsed, and mounted in medium containing *p*-phenylenediamine (1 mg/ml) as an antibleaching agent. Appropriate controls, including incubations with primary or secondary antibody alone, were negative. The specificity of the MPM-2 antibody for mitotic cells was further established by the positive reaction with mitotic cell proteins on immunoblots, while similar blots of interphase cell protein were negative (data not presented).

For extraction experiments, cells were lysed for 2 min in a Triton X-100-containing extraction buffer (PHEM buffer; pH 6.9) as described by Schliwa and van Blerkom (6). In addition, taxol (5 µg/ml) was added for additional stabilization of the microtubules (exposure of cells to this level of taxol for the times indicated has been demonstrated not to alter the microtubule network in these cells). Cells were rinsed and maintained in the above buffer without taxol or Triton X-100 for 30–45 min at room temperature. Cells were then fixed with 0.7% glutaraldehyde in P_i/NaCl and processed as above.

Periodate/lysine/paraformaldehyde fixative was prepared as described by McLean and Nakane (7), using a 2% final formaldehyde concentration. Fixation was for 12 min at room temperature, followed by a rinse in P_i/NaCl and cell lysis in 0.1% Triton X-100 for 2 min.

In the double-labeling experiments, coverslips were incu-

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bated in the following sequence: MPM-2 antibody, rhodamine-conjugated goat anti-mouse immunoglobulin, human anti-centrosomal antibody, and fluorescein-conjugated goat anti-human immunoglobulin. All conjugated antibodies were obtained from Cappel Laboratories (West Chester, PA).

Microscopy. Mounted coverslips were examined with a Zeiss Universal microscope equipped with epifluorescence optics using an X63 phase, 1.4 NA planapochromat objective. Kodak Tri-X film was used for photography.

RESULTS

Discrete Mitotic Structures Contain MPM-2 Immunoreactive Material. Immunofluorescent staining with a monoclonal antibody reactive with a subset of cellular phosphoproteins (5) was detectable in both interphase cells and mitotic cells. However, the intensity of the staining was significantly less in interphase cells and was restricted to discrete patches or spots within the nucleus (Fig. 1 *a* and *b*). It was clear from

FIG. 1. Monolayer cultures of Pt K1 cells stained by indirect immunofluorescence with monoclonal antibody MPM-2. Phase-contrast- and fluorescence-photomicrograph pairs of cells at interphase (*a* and *b*), prophase (*c* and *d*), prometaphase (*e* and *f*), metaphase (*g* and *h*), anaphase (*i* and *j*), and telophase (*k* and *l*). An example of a phase dense spot corresponding to the kinetochore and its corresponding immunofluorescent pair are indicated by the circles in *c* and *d*. (Bar = 10 μ m.)

comparison of the phase and fluorescence micrographs that none of the immunofluorescent spots corresponded to the nucleoli. Like interphase cells, cells in prophase showed no cytoplasmic staining, but the intensity within the nucleus was increased dramatically (Fig. 1 *c* and *d*). The area of the condensed chromosomes contained small bright spots associated with phase dense areas on the chromosomes themselves. The morphology of these phase dense spots in prophase, together with their appearance in other mitotic stages, suggested that the spot staining was located at the kinetochore. The term kinetochore as used here refers to the morphologically defined chromosomal constriction that serves as the region for spindle attachment.

At the time of nuclear envelope breakdown, the immunoreactive material previously retained within the nucleus appeared to be spread throughout the cytoplasm (Fig. 1 *e* and *f*). Concurrent with this event, strong antibody labeling was observed in the centrosomal region. The cells exhibited the greatest overall fluorescence at metaphase (Fig. 1 *g* and *h*). Over the general cytoplasmic background, staining was observed at the spindle poles, the kinetochores, and the spindle itself. The immunoreactive material at the spindle poles was detected from prometaphase until the cells entered early G₁ (Fig. 1 *f*–*i*), but the intensity of the staining decreased markedly after anaphase. Staining at kinetochores was no longer apparent by late anaphase. As the cleavage furrow became more pronounced, immunoreactive structures were detected in the area where polar microtubule overlap would be expected. These structures coalesced into a brightly staining region on either side of the phase dense matrix of the midbody (Fig. 1 *k* and *l*), but this staining was not distributed along the length of the microtubules extending out from the midbody (see below). It is also noteworthy that the nuclei of daughter cells in the process of cytokinesis did not contain the intranuclear spots and patches present in interphase cells. These developed as the two daughter cells completed cytokinesis in early G₁.

Cytoplasmic Immunoreactive Material Is Soluble. The containment of the immunoreactive material within the nucleus of prophase cells and the dispersal of the material throughout the cytoplasm after nuclear-envelope breakdown suggested

that much of the diffuse material was not associated with specific cytoplasmic structures. To test this suggestion and to better visualize the structural (insoluble) reactive material, we extracted cells with 0.15% Triton X-100 prior to fixation. To confirm that, under the extraction conditions used, the cells retained their normal pattern of microtubules, cells were also immunostained with monoclonal antitubulin.

When compared with nonextracted cells, cells lysed and washed extensively in PHEM buffer prior to fixation showed a similar localization of MPM-2 reactive material to mitotic organelles (Fig. 2 *a*–*d*). The most significant differences were in the relative intensity of the fluorescence in the mitotic stages following nuclear envelope breakdown. The general cytoplasmic background was greatly reduced, apparently because of the removal of soluble material, whereas the immunoreactive material was retained at the spindle poles, chromosomes, and midbody (Fig. 2 *c* and *d*). Direct comparison of a metaphase and interphase cell is also shown in Fig. 2 *c*. The most intensely staining structures are the spindle poles, and these are significantly brighter than any of the spots in the interphase nucleus. Not obvious in unextracted cells but apparent in extracted cells was a weak diffuse reaction of the condensed chromosomes with the MPM-2 antibody, superimposed on which was the spot staining at the kinetochores.

Comparison of the MPM-2 localization (Fig. 2 *a*–*d*) with that of tubulin in cells at the same position in the cell cycle (Fig. 2 *e*–*h*) demonstrated the integrity of the microtubule network under the extraction conditions used and also its relationship to the mitotic phosphoproteins. In relation to the microtubule pattern, MPM-2 differs in that it strongly stains mitotic centrosomes and kinetochores, does not stain the interphase network of microtubules, does not stain astral microtubules, appears to weakly stain kinetochore microtubules, and finally, does not stain the microtubules radiating away from the midbody but is restricted to a region adjacent to the midbody matrix where microtubules are thought to overlap (8).

Similar MPM-2 localization patterns were obtained with Chinese hamster ovary and HeLa cells. Lysis of Pt K1 cells with no extended washing period or washing of lysed Pt K1

FIG. 2. Immunofluorescence micrographs of Pt K1 cells stained with monoclonal antibody MPM-2 (*a*–*d*) or monoclonal antitubulin (*e*–*h*). Soluble immunoreactive material was removed prior to fixation by extraction of cells with a detergent containing microtubule-stabilizing buffer. (*a*) Interphase cells retain intranuclear MPM-2 reactive spots and patches. (*b*) Spot and patch staining also present in prophase nuclei. (*c*) Metaphase cell, upper right, demonstrates removal of the majority of the cytoplasmic fluorescence and retention of staining at the spindle poles, kinetochores, spindle fibers, and chromosomes. (*d*) Midbody structures retain immunoreactive material. (*e*) Typical interphase microtubule network. (*f*) Prominent centrosomal array of microtubules at prophase. (*g*) Metaphase mitotic spindle. (*h*) Reformation of cytoplasmic microtubule network and presence of midbody at telophase. (Bar = 10 μ m.)

cells for 5 min in PHEM buffer containing 2 mM Pipes (pH 6.9) or 0.5 M KCl did not alter the localization of MPM-2. The intensity of the MPM-2 staining was reduced approximately 50% by incubating the lysed PtK₁ cells for 30 min at 20°C with 10 units of alkaline phosphatase per ml in PHEM buffer as determined by comparison of negatives taken at identical exposure times (data not shown).

Immunoreactive Spots on Chromosomes Mark Kinetochores. The spot staining of condensed chromosomes suggested that MPM-2 reacted with the kinetochores, but the general diffuse staining of chromosomes hindered a clear-cut demonstration of this association. In exploring protocols for immunofluorescence, we found that cells prepared by using periodate/lysine/paraformaldehyde fixative gave the clearest images of fluorescence localization at the centrosome and kinetochores (Fig. 3 *a* and *b*). The prometaphase cell in Fig. 3*a* demonstrates the association of pairs of brightly staining spots with individual chromosomes, and the metaphase cell in Fig. 3*b* shows two sets of these spots oriented on each side of the forming metaphase plate. This evidence strongly indicates a localization to the kinetochores of the small spots. As in the extracted cells, a fibrous array is evident radiating away from the spindle poles toward the chromosomes.

Interphase Centrosomes Are Weakly Reactive. Although it was clear that the mitotic centrosome stained strongly, a similar conclusion could not be drawn with the MPM-2 antibody for the interphase centrosome. The interphase centrosome is known to be located near the nuclear periphery or overlapping with the nucleus, and multiple spots in the nucleus have been observed with the MPM-2 antibody, but the interphase centrosome is not generally identifiable in cells by phase microscopy; therefore, an association with any of the MPM-2 reactive spots could not be made. To test for such an association, a centrosomal marker was required, and for this purpose we used a human anti-centrosomal serum. Human foreskin fibroblasts were double-labeled with an antibody to human centrosomes and MPM-2 to distinguish the centrosome from other MPM-2-positive spots. Whether observed adjacent to or superimposed on the nucleus (Fig. 4 *a* and *d*, respectively), phase dense spots can be seen that coincide with the localization of the human anti-centrosomal antibody (Fig. 4 *b* and *e*). The MPM-2 antibody colocalizes to these spots in interphase cells. This can be clearly seen when the centrosome is adjacent to the nucleus as in Fig. 4*c*, but when the centrosome lies superimposed on the nucleus (Fig. 4*f*), this localization is obscured by the other MPM-2 reactive spots. The size of the MPM-2 reactive spot was con-

FIG. 3. Pt K1 cells fixed in periodate/lysine/paraformaldehyde and stained with monoclonal antibody MPM-2. Immunoreactive material is associated with kinetochores and centrosomes in both the prometaphase (*a*) and metaphase (*b*) cells. Two individual chromosomes with two associated immunoreactive spots are indicated by the arrowheads (*a*). Weak spindle fiber immunofluorescence remains (*b*), but cytoplasmic and chromosomal staining is absent. (Bar = 10 μ m.)

FIG. 4. Human foreskin fibroblasts were double stained with monoclonal antibody MPM-2 and human anti-centrosomal antibody. Phase-contrast and immunofluorescence micrographs of two cells show the centrosome when viewed adjacent to (*a-c*) or superimposed on (*d-f*) the nucleus. Centrioles are indicated by the arrowhead in *a* and *d*. The human anti-centrosomal antibody labels the respective centrosomes (arrowheads in *b* and *e*). The reaction of monoclonal antibody MPM-2 with the centrosome can be observed when the centrosome is adjacent to the nucleus (arrowhead in *c*) but cannot be distinguished from other nuclear spots when the centrosome is superimposed on the nucleus (*e*). (Bar = 5 μ m.)

sistently smaller than that of the human anti-centrosomal spot, suggesting that the MPM-2 antibody recognized centriolar proteins but not the full complement of interphase pericentriolar material.

DISCUSSION

The monoclonal antibody MPM-2, initially raised against HeLa mitotic cells, has been shown to react with a subset of cellular phosphoproteins particularly abundant in mitotic cells (5). We have shown here that, in addition to reacting with the nucleus of prophase cells and with soluble material in the cytoplasm of mitotic cells, it also reacts specifically with structural components of mitotic microtubule organizing centers, namely, centrosomes, kinetochores, and midbodies.

Immunoreactive material within the mitotic cytoplasm appeared to originate, at least in part, from the breakdown of the nuclear envelope, releasing soluble proteins previously contained within the nucleus. The immunoreactive material may have been generated also by phosphorylation of cytoplasmic proteins after the release of an essential factor normally sequestered within the nucleus.

Phosphorylation may play a role in the mechanism of nuclear envelope breakdown and formation of soluble nuclear

lamina proteins (3, 9). Recently, a model that links the activity of maturation-promoting factor with a phosphoprotein cascade resulting in increased phosphorylation of lamina proteins has been proposed (10). Mitotic factors identified in somatic cells may be involved similarly (11–13).

Extraction of the soluble material from mitotic cytoplasm permitted us to identify that centrosomes, kinetochores, and midbodies were also immunoreactive. This may mean that phosphorylation–dephosphorylation events are required for the formation, breakdown, and/or function of the mitotic spindle. We wish to note, however, that the timing of the association of immunoreactive material with the microtubule organizing centers need not signify the timing of the protein phosphorylation. The appearance of phosphorylated antigenic determinants within the microtubule organizing center could result from (i) direct phosphorylation of proteins within the preassembled microtubule organizing center, (ii) incorporation into the microtubule organizing center of proteins phosphorylated previously, or (iii) unmasking of preexisting determinants normally buried in the interphase microtubule organizing center. Such unmasking of antigenic determinants during mitosis has been demonstrated for both microtubule-associated protein (14) and cytokeratin filaments (15) in PtK cells. Unmasking of determinants is considered to be less likely in these studies because the phosphoprotein antigens are not detectable on NaDodSO₄/polyacrylamide gels of proteins from synchronized interphase HeLa cells (5). Similarly, the loss of immunoreactive material associated with the microtubule organizing centers could result from dephosphorylation of structural proteins, loss of phosphoproteins from the structure, or masking of antigenic determinants. Any combination of the above mechanisms could be responsible for the differential appearance and disappearance of immunoreactive material at a microtubule organizing center.

Other localized regions of MPM-2 immunoreactive material included the interphase nuclei. The nature of these nuclear spots and patches was not clear, but they were larger and more diffusely stained than are the kinetochore spots that appeared subsequently. Also, interphase prekinetochore spots, as detected by human autoantibodies from scleroderma patients (16), appeared more focused than the MPM-2 reactive material. They also were not associated with the nucleoli and were absent from the nuclei of daughter cells that had not completed cytokinesis. Cells in prophase showed a sharp increase in the intensity of nuclear staining that obscured the interphase nuclear patches, and their ultimate fate, therefore, could not be determined.

The pronounced peak in the amount of immunoreactive material in mitotic cells, and its specific association with the nucleus and microtubule organizing centers suggest that phosphorylation–dephosphorylation events may pleiotropi-

cally mediate the interphase–mitotic and mitotic–interphase transitions. However, other than the role phosphorylation may play in the mechanism of nuclear envelope breakdown, the functional significance of the mitotic phosphoproteins is not known. Of the more than 16 polypeptides recognized by the MPM-2 antibody (5), none of the individual proteins has been identified as a component of a specific mitotic structure. The immunostructural studies presented here indicate that some of these polypeptides are likely to be components of centrosomes, kinetochores, and midbodies. This is supported by the recent identification of four kinetochore phosphoproteins of 14, 15.5, 20, and 23 kilodaltons (17). It will be important not only to identify the possible crossreactivity of MPM-2 with these kinetochore polypeptides but also to determine the identity and the dependence of mitotic events on the phosphorylation state of components of the other mitotic microtubule organizing centers.

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