Mitosis-Specific Monoclonal Antibodies Block Cleavage in Amphibian Embryos

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ABSTRACT. By microinjecting monoclonal antibodies that bind specifically to mitotic and meiotic cells of a variety of species, we studied the biological activity of antigens recognized by these antibodies. The antibodies recognize a family of phosphoprotein antigens that are found throughout the cytoplasm of mitotic cells and particularly at microtubule organizing centers, including centrosomes and kinetochores. Their binding is dependent on phosphorylation of the polypeptides. Immunoglobulins were introduced into Xenopus laevis and Rana pipiens oocytes or cleaving embryos using glass micropipettes. The ability of the antibody-injected oocytes to undergo mitosis or meiosis was compared with those injected with control mouse immunoglobulins. The antibodies failed to block chromosome condensation and germinal vesicle breakdown in progesterone-treated oocytes. However, functional mitotic spindles were not assembled in cleavage stage frog embryos injected with antibodies. In vitro, the binding of the antibodies to the antigens inhibited the dephosphorylation of the antigens by alkaline phosphatase. The antibody binding to the activated microtubule organizing centers (MTOC) seems to block not only the nucleation of microtubules and the organization of the mitotic spindle, but also the dephosphorylation of proteins associated with the MTOC that normally occurs at the mitosis-G₁ transition.

There are proteins or activities present in mitotic cells that are absent from interphase cells. Indeed, cells will not enter mitosis if protein synthesis has been inhibited for more than 1 h (15, 21). Cells in mitosis can induce mitosis-like changes, including nuclear dissolution and chromosome condensation, in interphase cells to which they are fused (13, 16). Extracts from cells in mitosis (20, 24) or meiosis (14) can induce similar mitosis-like events when they are microinjected into immature frog oocytes.

Similarly, several antibodies that exhibit specific or increased binding to cells in mitosis compared with cells in interphase have been produced (4, 7, 8). Whether the antigens recognized by the antibodies have a direct role in the process of mitosis has not yet been demonstrated. For example, the antibodies used by Drahovsky and Kaul (8) react with DNA-cytosine-5'-methyltransferase and are thought to give increased immunohistochemical staining of cells in mitosis because of increased ac-

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cessibility of, or conformational changes in, the antigen at mitosis.

Our laboratory has described two monoclonal antibodies, MPM-1 and MPM-2, to a family of phosphopolypeptide antigens detectable by immunohistology specifically at mitosis (5, 6). Polypeptides of mitotic cells, but not interphase cells, react with the antibodies on immunoblots, showing that the increased staining is not due solely to accessibility. The antigens are found in all species so far examined, but always associated with mitotic or meiotic cells. Antigens dephosphorylated by bacterial alkaline phosphatase no longer react with these antibodies. These data are consistent with those of Adlakha et al. (1, 18), who showed that the phosphorylation and dephosphorylation of nonhistone proteins is specifically associated with entry into and exit from mitosis, and the work of Halleck et al. (10, 11), who detected a protein kinase activity only in mitotic cells. Vandre et al. (22, 23) have recently described the subcellular localization of the antigens: they are particularly apparent at microtubule organizing centers (MTOC), including centrosomes, kinetochores, and midbodies. Other studies supporting the hypothesis that these phosphoprotein antigens are functionally important in the process of mitosis are those of Hecht et al. (12), who reported that the antigens were absent at restrictive temperature in a temperature sensitive, embryonic arrest mutant of Caenorhabditis elegans. Upon shift to the permissive temperature the nematode cells rapidly entered mitosis; the antigen appeared concomitantly in 90% of the cells. Yamashita et al. (25) have reported similar observations using a mutant cell line of baby hamster kidney cells that exhibits nuclear membrane breakdown and premature chromosome condensation (PCC) at the restrictive temperature. The kinetics of appearance of PCC and the detection of antigens by immunohistological staining and on immunoblots in cells at the nonpermissive temperature were similar to those observed at the permissive temperature when cells go through normal mitosis. An abstract by Centonze et al. (3) reports that the growth of microtubules from isolated centrosomes is inhibited by MPM-2 antibodies.

In our previous studies we have shown that microinjection of MPM-1 and MPM-2 antibodies had no effect on the entry of HeLa cells into mitosis but their exit from mitosis to G1 was either delayed or totally blocked (5). The object of this study was to examine the mechanism by which these antibodies arrest the cells in mitosis. For this purpose we selected two-cell stage embryos of Xenopus and Rana for microinjection with the antibodies and study by thin sectioning of oocytes to determine the point of arrest in mitosis. These studies revealed that injection of MPM-1 and MPM-2 had no effect on chromosome condensation and nuclear membrane breakdown but prevented the formation of a functional spindle thus leading to metaphase arrest.

MATERIALS AND METHODS

Rana pipiens and Xenopus laevis oocytes were obtained by surgical dissection as described previously (20). Cleavage-stage embryos were obtained by in vitro fertilization of ovulated eggs (9, 17). After antibody injection, embryos were fixed, sectioned, and stained with Feulgen-Fast green as previously described (20).

MPM-1 and MPM-2 immunoglobulins were prepared by ammonium sulfate precipitation (19) of mouse ascites fluids from hybridoma clones prepared in our laboratory (6). Control mouse immunoglobulin was prepared from the ascites fluid produced by injection of the SP2/0 myeloma, which does not secrete immunoglobulin.
Frog oocytes and embryos were injected by using glass micropipettes as previously described (20).

**RESULTS**

*Antibodies do not inhibit germinal vesicle breakdown in Xenopus oocytes.* In order to investigate the biological function of the mitosis-specific phosphoprotein antigens, the effect of injection of antibodies MPM-1 and MPM-2 (2 mg/ml) either before or after incubation of frog oocytes with progesterone was compared with that of control mouse immunoglobulin. Neither of the antibodies was able to inhibit or delay the progesterone-induced GVBD that usually occurs within 8 h after progesterone treatment. All the oocytes (10 per each treatment) injected with either preimmune serum or MPM-1 or MPM-2 antibodies exhibited GVBD at the same time as controls.

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**Fig. 1.** MPM-2 inhibits cell division in cleaving frog embryos. Frog embryos at the 2-cell stage were injected in one hemisphere with approximately 65 ng of either MPM-2 or control mouse immunoglobulin. Further cleavage in the cells of the injected embryos was observed over 2–4 h after injection. Arrows indicate site of injection. *Rana pipiens* Embryos: (a) injected with control mouse immunoglobulin showing continued cleavage in both hemispheres, and (b) injected with MPM-2 immunoglobulin, showing continued cleavage only in the uninjected hemisphere. Histological sections of *Xenopus laevis* embryos: (c) control mouse IgG showing normal metaphase spindle with asters and chromosomes on the metaphase plate, (d) injected with MPM-2 showing condensed chromosomes in a cluster with no detectable mitotic apparatus.
time as the control uninjected oocytes.

Spindle formation and cleavage in frog embryos is inhibited by antibodies. The ability of the MPM-1 and MPM-2 immunoglobulins to inhibit cleavage in frog embryos was also investigated. *Rana pipiens* and *Xenopus laevis* embryos at the two-cell stage were injected into one of the cells with either control mouse IgG or MPM-2 IgG, and cleavage in the embryos was observed over the next 4–6 h. Most of the embryos injected with control mouse IgG (> 97%) showed continued cleavage in both hemispheres without any apparent delay (Fig. 1, panels a and b, and Table I). In contrast, only 4.8% or less of the embryos injected with MPM-2 IgG exhibited continued cleavage in both hemispheres. Most showed continued cleavage in the noninjected hemisphere, with no additional cleavage in the injected hemisphere. In some, no additional cleavage in either hemisphere was observed. In these, first cleavage may not have been complete at the time of injection. Sections of cells showing cleavage inhibition revealed condensed chromosomes with no apparent spindle in all 12 embryos examined by complete serial sectioning (Fig. 1 c, d). Thus, nuclear envelope breakdown and chromosome condensation was not inhibited by the antibodies, but spindle formation and subsequent steps in mitosis were inhibited. Cleavage continued in many uninjected cells through the 32-cell stage and beyond, yet did not resume in the antibody-injected cells, nor was there evidence for nuclear reformation.

Antibody binding inhibits dephosphorylation of antigens. Since MPM-1 and MPM-2 had previously been shown to bind to proteins only when those proteins were phosphorylated (6), the recognized antigenic epitope might be at or near the phosphorylated site. In addition, the total amount of antigen detected in cells began to decrease as early as anaphase-telophase (6). For this reason, the ability of the antibodies to inhibit dephosphorylation by alkaline phosphatase *in vitro* was investigated. Extracts from mitotic cells that had been prelabelled during G2 with $[^{32}P]$orthophosphate were incubated with either MPM-2 or control mouse IgG prior to treatment with alkaline phosphatase. Afterwards, the antigens were precipitated using MPM-2 and Pansorbin, and the amount of precipitated radioactivity was determined. Treatment with alkaline phosphatase, with no added IgG, decreased the amount of $[^{32}P]$ precipitated by 88% (Fig. 2). Treatment with control mouse IgG did not protect against the phosphatase digestion. When extracts were pretreated with MPM-2 IgG before alkaline phosphatase treatment, however, 85%

| Table 1. Inhibition of cell division in cleaving frog embryos by microinjection of MPM-2 |
|-----------------|-----------------|-----------------|
| Species         | Antibody injected | # Embryos with continued cleavage/# Embryos injected (%) |
| *Xenopus laevis*| Mouse serum Ig   | 97/97 (100.0)   |
|                 | MPM-2 Ig         | 4/83 (4.8)      |
| *Rana pipiens*  | Mouse serum Ig   | 62/64 (97.0)    |
|                 | MPM-2 Ig         | 2/61 (3.0)      |

Approximately 35 nl of immunoglobulin solutions at 2 mg/ml were injected into one cell after first cleavage. Embryos were observed over the next 4–6 h for continued cleavage of both the injected and uninjected cells.
Mitotic Arrest by Monoclonal Antibodies

Fig. 2. MPM-2 inhibits in vitro dephosphorylation of mitotic proteins by alkaline phosphatase. Aliquots (100 μg) of extract from synchronized mitotic cells that had been prelabelled with 32P-orthophosphate during the G2-phase were incubated for 0.5 h at 37°C and 1 h at 5°C with buffer alone (A); 100 μg of either control mouse IgG (B) or MPM-2 (C). Alkaline phosphatase (100 units/ml in 0.1 M Tris-HCl pH 8) was added to half of the aliquots to a final concentration of 10 units/ml, while buffer only was added to the remaining tubes. Digestion with alkaline phosphatase was for 0.5 h at 37°C. Subsequently, antigens were precipitated by the addition of 100 μg MPM-2 and 200 μl Pansorbin as described (6) and the amount pelleted radioactivity was determined. The amount of radio activity present in alkaline phosphatase-treated samples (solid bars) was expressed as per cent of the buffer treated controls (open bars).

of the label initially present could be precipitated. Thus, the MPM-2 IgG protected the antigens from dephosphorylation.

DISCUSSION

The monoclonal antibodies MPM-1 and MPM-2 have previously been shown to react with a subset of cellular phosphoproteins greatly enriched in mitotic and meiotic cells from a wide variety of species (6). Moreover, these antibodies react specifically with structural components of microtubule organizing centers, including centrosomes, kinetochores, and midbodies (22, 23). Both the specificity of the antibodies for mitotic and meiotic cells over a broad range of species and the localization of the antibodies in structures required for chromosome segregation suggested that the antigenic epitope recognized was functionally important in the mitotic process. By the experiments reported here we show that these antibodies are able to inhibit spindle assembly and function but not chromosome condensation and nuclear envelope breakdown. Since the antibodies are able to inhibit the alkaline phosphatase-induced dephosphorylation of the antigens in vitro, the spindle forma-
tion and completion of mitosis in vivo may be mediated by this mechanism.

The most significant effect of MPM-2 appears to be the arrest of cells in mitosis. About 70% of HeLa cells synchronized in mitosis by N2O blockade and injected with MPM-2 are able to exit from mitosis upon reversal of the block, although the mitotic index remains at 20% even at 20 h after reversal (5). About 95% of cleaving frog embryos injected with MPM-2 fail to continue cell division. Furthermore, histological sections showed that the cleavage-arrested cells contained condensed metaphase chromosomes with no apparent spindle. What is the mechanism of mitotic blockade by MPM-2?

The injection of MPM-2 into progesterone-stimulated oocytes may prevent spindle formation due to interference with the activity at MTOC, that contain increased concentrations of MPM-2 reactive material when they are activated for spindle formation. This explanation is consistent with the observation of Centonze et al. (3), who showed that MPM-2 was able to inhibit microtubule nucleation by isolated centrosomes in vitro. Therefore, the metaphase arrest observed in the embryos was due to the absence of a functional spindle. This may be the first example where a monoclonal antibody has been shown to inhibit spindle formation. The mitotic arrest induced by the injection of MPM-1 and MPM-2 could be due, not only to the failure of spindle organization but also the inhibition of protein dephosphorylation necessary for mitosis to G1 transition as indicated in our earlier studies (1, 2, 18).

Added in Proofs: Our latest studies reveal that the monoclonal antibody MPM-2, when it is highly purified and concentrated (20 mg/ml, i.e., about 10 times more concentrated than that used in this study), is capable of not only inhibiting progesterone-induced maturation of Xenopus oocytes but also neutralize as well as immunodeplete M-phase promoting factor (MPF) activity from mitotic HeLa cell and mature oocyte extracts. [Kuang, J., Zhao, J-Y., Wright, D. A., Saunders, G. F. and Rao, P. N. Proc. Natl. Acad. Sci. USA. 1989 (in press)]

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