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Ca^{2+}/calmodulin-dependent protein kinase II: Localization in the interphase nucleus and the mitotic apparatus of mammalian cells

(mitosis/microtubules/protein phosphorylation/nucleolus)

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ABSTRACT **Indirect immunofluorescence was used to determine the distribution of Ca^{2+}/calmodulin-dependent protein kinase II (CaM kinase II) in rat embryo fibroblast 3Y1 cells, rat C6 glioma cells, and human epidermoid carcinoma KB cells. During interphase at growing phase, CaM kinase II was localized diffusely in the cytoplasm and in the nucleus. In the nucleus, the enzyme was localized within the whole nuclear matrix in which the enzyme was specially concentrated in nucleoli. During mitosis, CaM kinase II was found to be a dynamic component of the mitotic apparatus, particularly present at microtubule-organizing centers. In metaphase and anaphase, CaM kinase II was observed at centrosomes and between the spindle poles. During telophase, CaM kinase II was condensed as a bright fluorescent dot at the midzone of the intercellular bridge between two daughter cells, while tubulin was found at each side of the midbody. Colchicine, a microtubule inhibitor, disorganized the tubulin- and CaM kinase II-specific fluorescent structure of mitotic 3Y1 cells. In cold-treated cells, CaM kinase II was localized predominantly at centrosomes. The localization of CaM kinase II in the cell nucleus and the mitotic apparatus suggests that the enzyme may play a role in the cell cycle progression of mammalian cells.**

A variety of studies have suggested that protein phosphorylation and dephosphorylation play important regulatory roles in the control of cell growth and cell cycle progression (1). Much attention to the role of protein phosphorylation has been focused on the two phases of the cell cycle—namely, the signal transduction of growth factors into the nucleus and the initiation and completion of mitosis (2).

The binding of growth factors such as epidermal growth factor and platelet-derived growth factor to their specific cell surface receptors initiates certain sets of protein phosphorylation. These include growth factor receptor-coupled activation of tyrosine-specific protein kinases, phosphatidylinositol turnover-coupled activation of protein kinase C, and Ca^{2+} mobilization-coupled activation of Ca^{2+}/calmodulin-dependent protein kinases. However, the linkage between the activation of protein kinases and the expression of corresponding nuclear events is largely unclear.

In the mitotic phase of the cell cycle, a large number of proteins are specifically phosphorylated (3). Mitotic events include various cellular processes, and they have been suggested to be regulated in part by protein phosphorylation. Davis *et al.* (4) have prepared a monoclonal antibody that recognizes a set of phosphoproteins abundant in mitotic cells. These phosphoproteins are associated with microtubule-organizing centers such as centrosomes, kinetochores, and midbodies (5). Spindle reactivation (anaphase B) in diatomes has been demonstrated to be correlated with the phosphorylation of a 205-kDa spindle-associated protein (6). Ca^{2+}/calmodulin-dependent phosphorylation of a 62-kDa protein induces microtubule depolymerization in sea urchin mitotic apparatus (7). These results suggest that phosphorylation of many of the proteins is associated with the reorganization of the cytoskeleton and the mediation of the microtubule-based intracellular motility during mitosis.

Ca^{2+}/calmodulin-dependent protein kinase II (CaM kinase II) is suggested to be involved in the regulation and coordination of various cellular processes (8, 9). A class of the kinase is identified in brain and other tissues and appears to have a relatively broad substrate specificity *in vitro*. Recently we identified CaM kinase II in rat embryo fibroblast 3Y1 cells using the affinity-purified, monospecific anti-CaM kinase II antibody (10). The enzyme has a major 50-kDa subunit and undergoes autophosphorylation in a Ca^{2+}- and calmodulin-dependent manner *in vivo*; this suggested that the kinase may mediate at least some of the biological actions of growth factors. In an effort to examine the role and distribution of CaM kinase II during the cell cycle of rat 3Y1 cells and other mammalian cells, we report the localization of CaM kinase II in the nucleus of interphase cells and the mitotic apparatus of mitotic cells.

MATERIALS AND METHODS

Materials. Fetal calf serum was obtained from Flow Laboratories; Eagle's minimal essential medium, from Nissui Seiyaku (Tokyo); [γ-^{32}P]ATP, from New England Nuclear; fluorescein isothiocyanate-conjugated IgG fraction from goat antiserum directed against rabbit IgG, from Cappel Laboratories; mouse monoclonal antibody (IgM) against calf brain tubulin, from Transformation Research, ICN; rhodamine-conjugated IgG fraction from goat antiserum directed against mouse immunoglobulin, from Cooper Biomedical; and penicillin G and kanamycin, from Meiji Seika (Tokyo). The affinity-purified rabbit anti-CaM kinase II antibody was prepared as described (10).

Cells. 3Y1 cells derived from Fischer rat embryo fibroblasts, rat C6 glioma cells, and human epidermoid carcinoma KB cells were grown at 37°C in tissue culture dishes in Eagle's minimal essential medium supplemented with 10% (vol/vol) fetal calf serum, 100 units of penicillin G per ml, and 0.2 mg of kanamycin per ml. For indirect immunofluorescence, the cells were grown in Corning 35-mm plastic culture dishes (Iwaki Glass, Tokyo) equipped with a 22 × 24-mm glass coverslip per dish.

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Abbreviation: CaM kinase II, Ca^{2+}/calmodulin-dependent protein kinase II.

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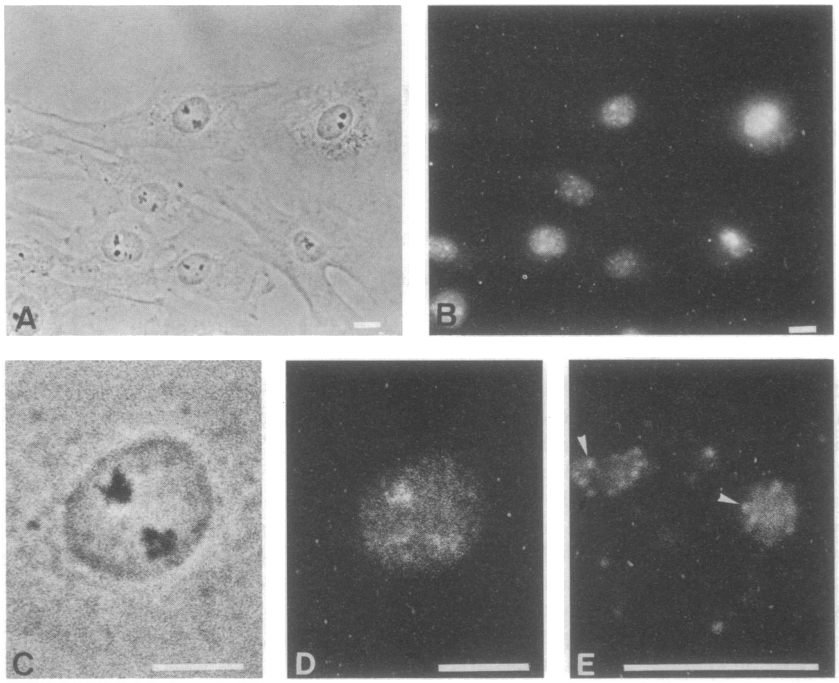

FIG. 1. Localization of CaM kinase II in interphase nuclei and nucleoli. Exponentially growing 3Y1 cells were examined by phase-contrast (*A* and *C*) and immunofluorescence (*B* and *D*) microscopy after being stained with affinity-purified anti-CaM kinase II antibody. (*E*) Immunofluorescence micrograph of the interphase 3Y1 cell nucleus with a higher magnification. CaM kinase II was distributed uniformly and concentrated on punctate structures (arrowheads), which are associated with the nucleolus. (Bars = 10 mm.)

Indirect Immunofluorescence. Cells grown on coverslips were routinely fixed with 3.7% formaldehyde in phosphate-buffered saline (PBS) at 37°C for 10 min, washed in PBS three times for 10 min, and treated with 0.5% Triton X-100 in PBS at 37°C for 10 min. Other fixation protocols used include: (*i*) methanol for 5 min at −20°C and (*ii*) 80% methanol/20% acetone (vol/vol) for 5 min at −20°C. Cells were washed three times more in PBS for 10 min and then incubated with 50 ml of 0.1% goat IgG in PBS containing 5% fetal calf serum in a moisture chamber at 37°C for 30 min. Then cells were washed again in PBS and subsequently incubated with primary antibodies (50 mg of the affinity-purified anti-CaM kinase II antibody per ml) at 37°C for 60 min. After three 10-min washes in PBS, samples were incubated with fluorescein isothiocyanate- or rhodamine isothiocyanate-conjugated second antibodies (goat anti-rabbit IgG or goat anti-mouse immunoglobulin)

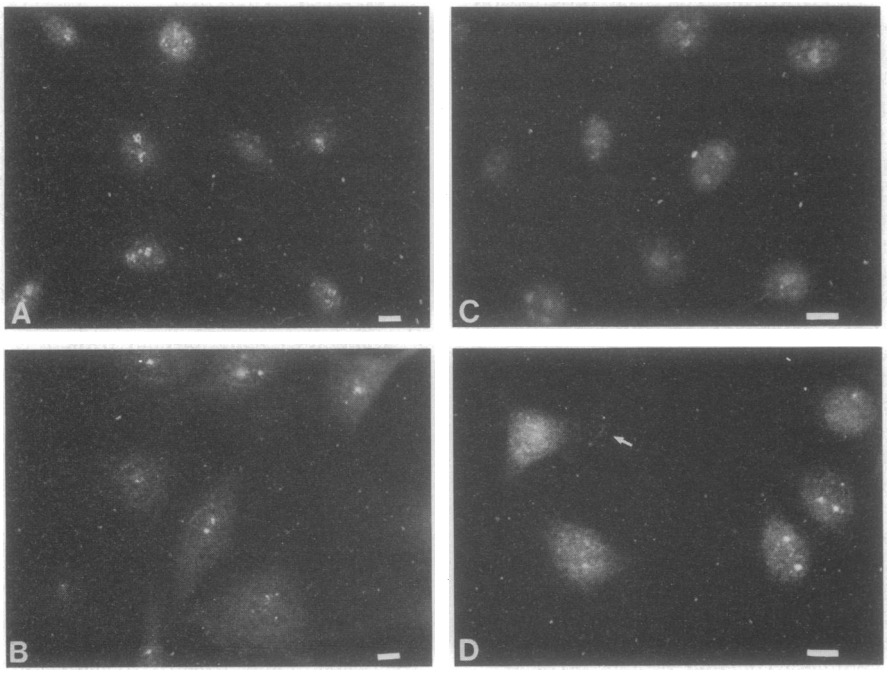

FIG. 2. Localization of CaM kinase II in interphase rat C6 glioma cells and human epidermoid carcinoma KB cells. Rat C6 glioma cells (*A* and *B*) and human epidermoid carcinoma KB cells (*C* and *D*) either were fixed in formaldehyde followed by extraction with detergent (*A* and *C*) or were fixed in 80% methanol/20% acetone (*B* and *D*). Fixed cells were incubated with affinity-purified anti-CaM kinase II antibody and processed for immunofluorescence. CaM kinase II was concentrated in some portions of the cell periphery (*D*, arrow). (Bars = 10 mm.)

at 37°C for 30 min. After three 10-min washes, the coverslips were mounted in PBS containing 50% (vol/vol) glycerol. Cells were observed and photographed with a Nikon Optiphoto microscope (Nikon) equipped with an EF illumination system with a 50-W mercury lamp (OSRAM). Images were recorded on Kodak Tri-X film. Controls for CaM kinase II immunofluorescence consisted of cells incubated with (*i*) second antibody only, (*ii*) IgG of the pass-through fraction of the respective antigen affinity column (500 μg/ml), (*iii*) IgG from nonimmune animals (3 mg/ml), or (*iv*) antibodies preabsorbed with antigen [50 mg of IgG per ml with a 4-fold molar excess of antigen (750 mg/ml)].

Treatment of Cells. Just before formaldehyde fixation and preparation for immunofluorescence, some cells were treated on coverslips as follows: (*i*) 1-hr incubation of cells with 1.0 mM colchicine at 37°C or (*ii*) 1-hr incubation of cells at 0°C.

RESULTS

Localization of CaM Kinase II in Interphase 3Y1 Cells. The affinity-purified, monospecific anti-CaM kinase II antibody recognized a 50-kDa protein in the whole extract of 3Y1 cells, and the 50-kDa protein immunoprecipitated with the antibody underwent autophosphorylation in a Ca^{2+}- and calmodulin-dependent manner *in vitro* (10). The specificity of the antibody was generally examined as previously described (10). 3Y1 cells were fixed with formaldehyde, permeabilized by exposure to detergent (Triton X-100), and incubated with the affinity-purified anti-CaM kinase II antibody for immunofluorescent localization of the proteins. Fig. 1 shows phase-contrast and immunofluorescence micrographs of nonsynchronized, exponentially growing 3Y1 cells labeled with the anti-CaM kinase II antibody. Comparison of the immunofluorescent staining pattern with the phase-contrast microscopy revealed that CaM kinase II appeared to be distributed diffusely throughout the nucleus (Fig. 1 *A* and *B*). The magnification of the nucleus and the comparison with the phase-contrast microscopy showed that CaM kinase II was localized within the whole nuclear matrix and was associated mainly with nucleoli (Fig. 1 *C* and *D*). Close examination of nucleoli revealed that CaM kinase II was concentrated in a dot structure (Fig. 1*E*, arrowheads). CaM kinase II did not appear to be associated with any discrete, subcellular structures in the cytoplasm. The enzyme was shown to be unassociated with cytoplasmic microtubules or stress fibers by comparison with anti-tubulin antibody

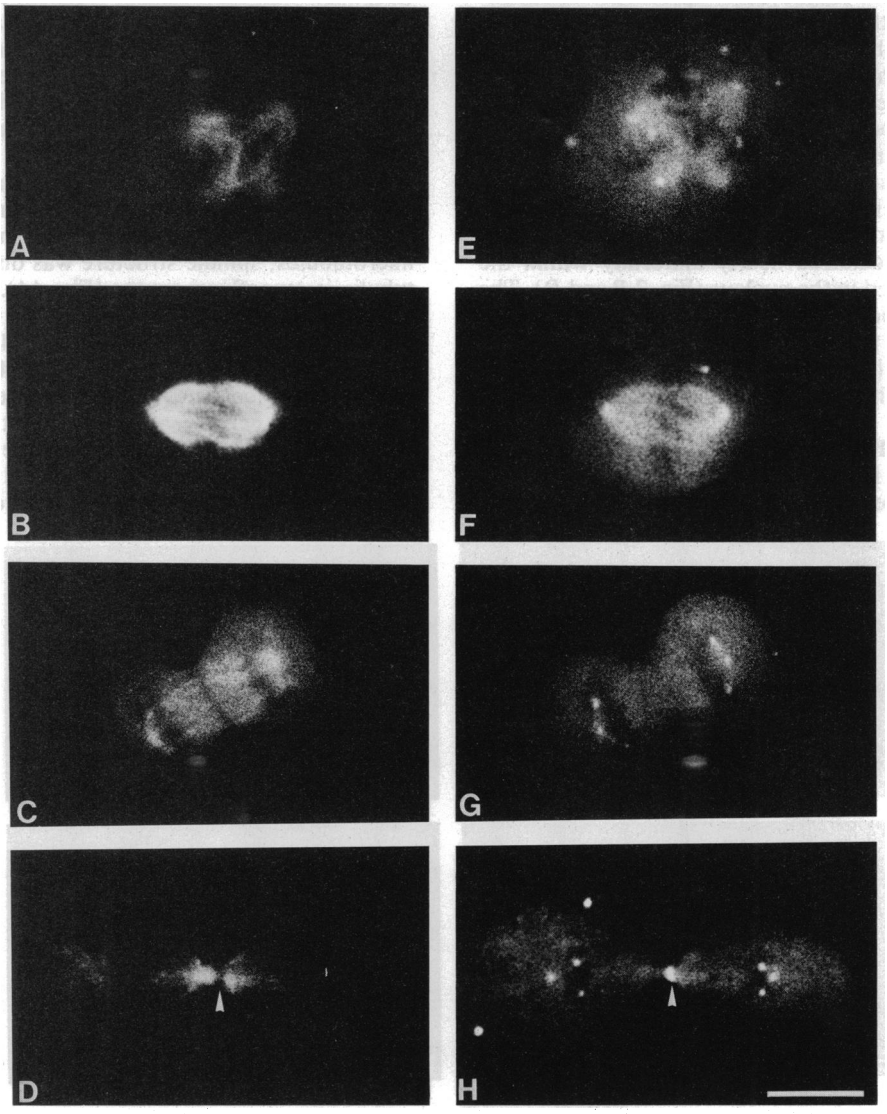

FIG. 3. Localization of tubulin or CaM kinase II in 3Y1 cells at different stages of mitosis. Identical cells were double-stained by immunofluorescence with anti-tubulin (*A–D*) and affinity-purified anti-CaM kinase II (*E–H*) antibodies at each stage of mitosis. (*A* and *E*) Prophase or prometaphase cell. (*B* and *F*) Metaphase cell. (*C* and *G*) Late anaphase or telophase cell. (*D* and *H*) Telophase cell. The midbody (*H*, arrowhead), which was immunostained with anti-CaM kinase II antibody, did not show immunofluorescence with anti-tubulin antibodies (*D*, arrowhead). (Bar in *H* = 10 mm.)

fluorescence or rhodamine-conjugated phalloidin fluorescence in interphase 3Y1 cells (data not shown). The specificity of the immunofluorescence was tested by performing four controls (see *Materials and Methods*). The background fluorescence was quite low, with only a trace of nonspecific fluorescence (data not shown).

Localization of CaM Kinase II in Interphase Rat C6 Glioma Cells and Human KB Cells. To examine if the nuclear localization of CaM kinase II seen in interphase rat fibroblast 3Y1 cells is observed in other mammalian cell types, we further studied the distribution of CaM kinase II in rat C6 glioma cells and human epidermoid carcinoma KB cells. Cytoplasmic extracts of the C6 cells and KB cells were separately prepared and immunoprecipitated with the affinity-purified anti-CaM kinase II antibody. Then the immunoprecipitates were incubated with 50 mM [γ-^{32}P]ATP in the presence or absence of Ca^{2+} and calmodulin. The 50-kDa protein in both cell extracts was immunoprecipitated and phosphorylated in a Ca^{2+}- and calmodulin-dependent manner (data not shown). Since the major subunits of purified CaM kinase II from various tissues are ≈50-kDa proteins and, moreover, since Ca^{2+}- and calmodulin-dependent autophosphorylation is a common property of the isoenzymes, the results are consistent with the idea that the C6 cells and KB cells contain a class of CaM kinase II and that their major subunits are the 50-kDa proteins (10). Fig. 2 shows the localization of CaM kinase II in the C6 cells and the KB cells by indirect immunofluorescence. In both cell types, CaM kinase II was localized in the nucleus, as observed in 3Y1 cells, when cells were fixed with formaldehyde and permeabilized with the detergent (Fig. 2 *A* and *C*). On the other hand, when cells were fixed with methanol/acetone without the detergent lysis procedure, CaM kinase II was localized nearly uniformly within the cytoplasm and localized in the nucleus (Fig. 2 *B* and *D*). The enzyme appeared to be concentrated in some portion of the cell periphery, which may correspond to membrane ruffles (Fig. 2*D*, arrow). Similar results were obtained in the case of the 3Y1 cells fixed with methanol/acetone or methanol alone (data not shown).

Localization of CaM Kinase II During Mitosis. The distribution of CaM kinase II and tubulin in the mitotic apparatus of 3Y1 cell was studied. 3Y1 cells were fixed in formaldehyde, permeabilized by exposure to detergent, and incubated with the affinity-purified anti-CaM kinase II and/or anti-tubulin antibodies for immunofluorescent localization of the proteins.

At prometaphase, tubulin and CaM kinase II showed similar localization in 3Y1 cells (Fig. 3 *A* and *E*). Specific fluorescence of CaM kinase II appeared to be in the centriole and to be more prominent in the spindle at metaphase. At metaphase, CaM kinase II appeared to be distributed in the entire spindle (Fig. 3*F*). At anaphase or early telophase, tubulin was apparently localized in both the interzone (interpolar fibers) and the half-spindle (Fig. 3*C*), while CaM kinase II appeared to be concentrated near the poles (Fig. 3*G*). During cytokinesis, CaM kinase II was not localized in the cleavage furrow. In the two new daughter cells, CaM kinase II abruptly appeared at the midbody region (Fig. 3*H*, arrowhead), while tubulin was found to be present at each end of the midbody region (Fig. 3*D*). The immunostaining of CaM kinase II was observed to some extent in the cytoplasm surrounding the spindle during all stages of mitosis. Although fluorescence was generally diffuse, punctate fluorescence was shown somewhere during metaphase (Fig. 3*F*) and telophase (Fig. 3*H*). Similar localization of the enzyme was observed during mitosis of rat C6 glioma cells and human KB cells (data not shown). The distribution of CaM kinase II in mitotic apparatus was similarly observed with all the fixation protocols used for the 3Y1, C6, and KB cells (data not shown). The specificity of immunofluorescence reaction was confirmed by the control experiments (data not shown).

Effects of Colchicine and Low Temperature (0°C) on Tubulin and CaM Kinase II Localization. When mitotic 3Y1 cells were treated with colchicine, an agent that induces disassembly of microtubules, spindle structure was disrupted, as shown by tubulin immunofluorescence (Fig. 4*A*). Similarly, the distribution of CaM kinase II was correlated with the change of the mitotic apparatus (Fig. 4*C*). In Fig. 4*A*, the bright spot is assumed to be the centrosome (Fig. 4*A*, arrow), and CaM kinase II was localized in the structure (Fig. 4*C*, arrow).

When 3Y1 cells were incubated at 37°C, spindles with many pole-to-pole microtubules were observed (Fig. 3*B*). However, when cells were incubated at 0°C for 1 hr, the cold-labile, pole-to-pole microtubules disappeared and only

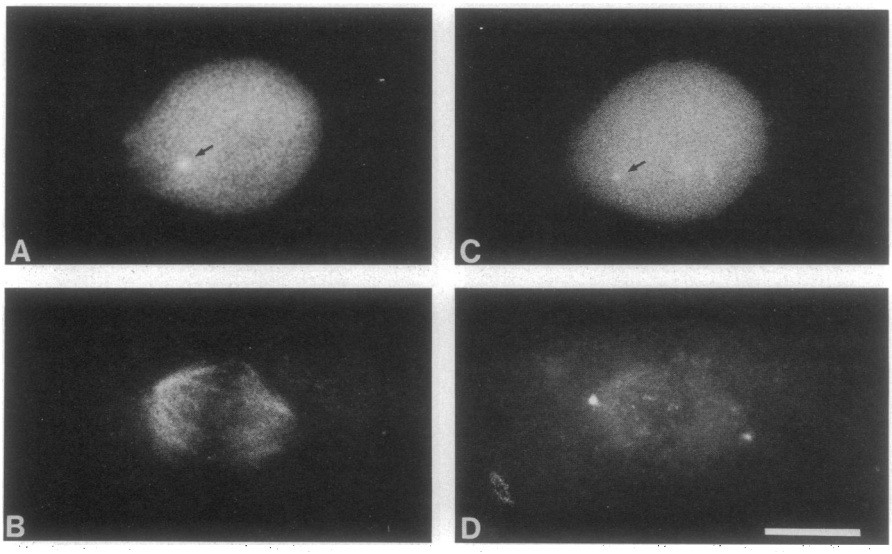

FIG. 4. Immunofluorescence localization of tubulin or CaM kinase II in metaphase 3Y1 cells. Identical cells were double-stained by immunofluorescence with anti-tubulin (*A* and *B*) and affinity-purified anti-CaM kinase II (*C* and *D*) antibodies. The 3Y1 cell was treated with 1.0 mM colchicine at 37°C for 1 hr (*A* and *C*) or incubated at 0°C for 1 hr just before being fixed and processed for immunofluorescence. The immunofluorescence of the spindle of tubulin and CaM kinase II was abolished by colchicine treatment. CaM kinase II was localized in a dot structure (*C*, arrow), and tubulin was similarly immunostained (*A*, arrow). Note the localization of CaM kinase II on the centrosomes when the cell was cold-treated. (Bar in *D* = 10 mm.)

the cold-stable, pole-to-pole chromosome microtubules remained (Fig. 4*B*). The concentration of CaM kinase II in centrosomes was still observed in cold-treated cells (Fig. 4*D*).

DISCUSSION

In the present report, we investigated the distribution of CaM kinase II in rat fibroblast 3Y1 cells, rat C6 glioma cells, and human epidermoid carcinoma KB cells during the cell cycle. In interphase 3Y1 cells, CaM kinase II was localized in the nucleus. In the nucleus, CaM kinase II was distributed within the whole nuclear matrix in which immunofluorescence was specially concentrated in nucleoli. In view of the fact that there are several substrates for a class of CaM kinase II in the neuronal nucleus (11) and that CaM kinase II may mediate some of the biological actions of growth factors in 3Y1 cells (10), CaM kinase II may transduce some of the Ca^{2+} signals into the nucleus (12). Furthermore, in the present study, CaM kinase II was found to be specially localized in the nucleolus. It also has been reported (13) that myosin light chain kinase occurs in the nucleolus. Further studies will be required to evaluate the functional significance of Ca^{2+}/calmodulin-dependent protein kinases associated with the nucleolus.

When 3Y1 cells, C6 glioma cells, and human KB cells were either fixed in methanol/acetone or methanol, CaM kinase II was found to be localized diffusely in the cytoplasm and the nucleus and to be concentrated in some portions of the cell periphery. However, when cells were fixed in formaldehyde and permeabilized by exposure to detergent, the enzyme was mainly localized in the nucleus. These results suggest that the soluble kinase was distributed nearly uniformly in the cytoplasm and that at least some of the particulate kinase that resists detergent extraction is tightly associated with the nuclear matrix of rat 3Y1 cells and other rat C6 glioma cells and human KB cells.

The localization of CaM kinase II was examined during mitosis in comparison to that of tubulin. Both proteins were found to be components of the mitotic apparatus. Furthermore, the localization of calmodulin in the mitotic apparatus was shown to be similar to CaM kinase II (14, 15). Several lines of evidence suggest that depolymerization of microtubules at anaphase is caused at least in part by phosphorylation of microtubule-associated proteins (MAPs) by CaM kinase II. (*i*) CaM kinase II inhibits microtubule assembly by phosphorylation of MAPs (16) and abolishes microtubule cold stability *in vitro* (17). (*ii*) Several MAPs were identified in the mitotic spindle (18, 19). (*iii*) In the mitotic apparatus of sea urchin, the Ca^{2+}/calmodulin-dependent phosphorylation of a 62-kDa protein was reported to induce depolymerization of microtubules (7). Under these conditions, CaM kinase II may be activated as follows. CaM kinase II undergoes autophosphorylation in the presence of Ca^{2+}/calmodulin and is converted to a Ca^{2+}/calmodulin-independent form *in vitro* (20–22). This may lead to the active state of the enzyme during anaphase even though the rise of the Ca^{2+} concentration is transient during metaphase (23).

At telophase, CaM kinase II was seen at the midbody region. This location was distinct from that of tubulin (Fig. 3*D*) and calmodulin (14, 15), since tubulin and calmodulin were observed at both sides of the midbody and not localized at the midbody region. In view of the presence of CaM kinase II in postsynaptic densities, CaM kinase II at the midbody may be integrated into the membrane and may play a role as a component of the membrane (24). Wordeman and Cande (6) have reported that the reactivation of the spindle in diatom is correlated with the phosphorylation of a 205-kDa protein in the spindle midzone. Protein phosphorylation may be involved in the activity of the spindle such as elongation.

When 3Y1 cells were treated with colchicine, similar changes in localization at metaphase occurred for both tubulin and CaM kinase II, suggesting that CaM kinase II is associated with microtubules. Since the cold treatment greatly reduced the distribution of CaM kinase II in the spindle, CaM kinase II may be associated mainly with centrosomes and cold-labile microtubules.

The present results demonstrate that CaM kinase II is the component of the centrosome. It is clearly exhibited in cold-treated cells. Since the centrosome is considered to control the number, spatial orientation, and length distribution of microtubules grown from itself (25), and phosphoproteins were shown to be associated with the centrosomes in metaphase cells (5), CaM kinase II may play a role in the regulation of centrosome functions through phosphorylation of the associated proteins.

It is generally accepted that the protein phosphorylation is essentially involved in the control of cell mitosis (2). Several protein kinases such as a homolog of the fission yeast *cdc2*-encoded protein kinase (26) and $pp60^{c\text{-}src}$ (27) are activated during mitosis and cdc2 kinase has been demonstrated to be associated with centrosomes during mitosis (28). The present results suggest that spindle function and microtubule dynamics during mitosis are regulated at least in part by CaM kinase II.

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