

# IDENTIFICATION OF ENZYMATICALLY ACTIVE $\text{Ca}^{2+}$ /CALMODULIN-DEPENDENT PROTEIN KINASE IN CENTROSOMES OF HEMOPOIETIC CELLS

Salvatore F. Pietromonaco<sup>1</sup>, Gustavo A. Seluja, Laurence Elias

Department of Medicine and the Cancer Center, University of New Mexico School of Medicine, Albuquerque, New Mexico, USA 87131.

Submitted on 02/13/95

(Communicated by P.W. Majerus, M.D., 02/21/95)

**ABSTRACT.** In this study, we report the identification of enzymatically active, multifunctional calcium/calmodulin-dependent protein kinase in centrosomes of FDCP1 cells using subcellular fractionation and immunofluorescence techniques. Centrosomes were isolated from detergent lysates of FDCP cells by sucrose density gradient centrifugation and contain tubulin ( $M_r = 58$  kDa) and centrin ( $M_r = 20$  kDa) by immunoblotting. Analysis of these fractions with anti-calcium/calmodulin kinase II antibody revealed the presence of the 52 kDa and 56 kDa doublet corresponding to the  $\alpha$  and the  $\beta/\beta'$  subunits of the enzyme complex. In vitro kinase reactions with isolated centrosomes and in the presence of calcium and calmodulin results in the phosphorylation of several centrosomal proteins.

Keywords: hemopoietic cells, protein kinases, centrosomes, calcium, calmodulin

## INTRODUCTION

The multifunctional calcium/calmodulin-dependent protein kinase ( $\text{Ca}^{2+}$ /CaM kinase II) is an important effector of  $\text{Ca}^{2+}$ /calmodulin signalling in cells. The enzyme complex is a multimeric structure (550-650 kDa) consisting of four ( $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$ ) closely related gene products of 56-60 kDa that become autophosphorylated in response to increases in intracellular  $\text{Ca}^{2+}$ .  $\text{Ca}^{2+}$ /CaM kinase II is most abundant in brain, where it functions to regulate neurotransmitter release and in the induction of long-term potentiation (1).

It is well documented that  $\text{Ca}^{2+}$  is required for cell cycle progression and mediates its actions via CaM, the primary intracellular receptor (2). In

quiescent rat embryo fibroblasts that are stimulated with serum to re-enter the cell cycle, a transient rise in intracellular  $\text{Ca}^{2+}$  activates  $\text{Ca}^{2+}$ /CaM kinase II, suggesting that the enzyme mediates the action of growth factors (3). Transient increases in  $\text{Ca}^{2+}$  are required for  $G_1$ -S and  $G_2$ -M phase progression (4). In a hemopoietic system in which buoyant tonsillar B cells are transiting the cell cycle,  $\text{Ca}^{2+}$ /CaM kinase II is more active than in resting, dense tonsillar cells (5). In a hairy cell leukemic cell line, which contains unusually high levels of intracytoplasmic  $\text{Ca}^{2+}$ , high levels of  $\text{Ca}^{2+}$ /CaM kinase II activity result in hyperphosphorylation of CD20, a membrane protein involved in B cell activation (6).

A role for  $\text{Ca}^{2+}$ /CaM kinase II in cell cycle

---

Abbreviations used: CaM, calmodulin; ECL, enhanced chemiluminescence; EDTA, ethylene diamine-N,N,N',N'-tetraacetic acid; EGTA, ethylene glycol-bis-(2-aminoethyl ether)-N,N,N',N'-tetraacetic acid; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; PBS, phosphate-buffered saline; PIPES, piperazine-N,N'-bis[2-ethanesulfonic acid]; SDG, sucrose density gradient; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TBS, Tris-buffered saline; TCA, trichloroacetic acid; W7, N-(6-aminohexyl)-5-chloro-1-naphthalene-sulfonamide.

<sup>1</sup> Reprint requests to: Salvatore F. Pietromonaco, Ph.D., University of New Mexico Cancer Center, 900 Camino de Salud, Albuquerque, NM, USA 87131. phone (505) 277-8532, fax (505) 277-2841.

progression is further supported by immunofluorescence studies localizing the enzyme (7) as well as CaM (8) in the mitotic apparatus and centrosomes of mammalian cells. A putative substrate of Ca<sup>2+</sup>/CaM kinase II has been localized to the mitotic apparatus and becomes phosphorylated in response to Ca<sup>2+</sup> and CaM (9). Furthermore, a 20 kDa Ca<sup>2+</sup>-binding protein named centrin or caltractin has extensive structural homology with CaM and is a component of the pericentriolar material in centrosomes from divergent species (10, 11).

Mouse leukemic FDCP1 cells are a growth factor-dependent cell line in which cell cycle progression can be studied. We became interested in studying Ca<sup>2+</sup>/CaM kinase II as part of our studies on the regulation of centrosome function by signal transduction pathways in hemopoietic cells. No studies to date have directly demonstrated the presence of enzymatically active Ca<sup>2+</sup>/CaM kinase II in centrosomes. In this report, we demonstrate that in FDCP1 cells Ca<sup>2+</sup>/CaM kinase II is associated with centrosomes using both immunofluorescence localization and subcellular fractionation. Furthermore, we demonstrate that the centrosomal Ca<sup>2+</sup>/CaM kinase II can be activated by Ca<sup>2+</sup> and CaM.

## METHODS

**Antibodies.** Affinity-purified antibody against Ca<sup>2+</sup>/CaM kinase II (RU-16) was obtained from A. Czernik, Rockefeller University, New York, NY. RU-16 was raised to a peptide corresponding to residues 501-531 of the  $\beta$  subunit that is highly conserved in all known subunits and recognizes the  $\alpha$  and  $\beta/\beta'$  subunits (12). Antiserum 26/14-1 against the pericentriolar protein centrin was obtained from J. Salisbury, Mayo Clinic, Rochester, MN. This antibody was raised against a *trpE*-centrin fusion protein expressed from a cDNA encoding *Chlamydomonas reinhardtii* centrin and reacts with mammalian centrin (10). Anti- $\alpha$  tubulin was obtained from Amersham (Arlington Heights, IL).

**Immunofluorescence.** FDCP1 cells ( $1 \times 10^6$ ) were pelleted from culture medium and washed twice with PBS. The cells were resuspended in 0.05 ml PBS and applied to poly-L-lysine-coated glass microscope slides. The cells were allowed to adhere for 15 min at 37°C, rinsed in PBS and then fixed in acetone for 3 min. The cells were rehydrated with PBS containing 1% BSA and incubated for 30 min at 37°C with rabbit anti-Ca<sup>2+</sup>/CaM kinase II (2  $\mu$ g/ml) and mouse anti-centrin (1:500) antibodies diluted in the same buffer. The slides were rinsed in PBS to remove unbound antibodies and incubated with goat anti-rabbit IgG (Fab')<sub>2</sub>-FITC (Zymed, South San Francisco, CA) and sheep anti-mouse IgG (Fab')<sub>2</sub>-Texas Red (Cappel, West Chester, PA) each diluted 1:400 in PBS/1% BSA. After additional washes in PBS, the slides were mounted in 2.5 mg/ml DABCO [1,4-diazobicyclo-(2,2,2)-octane] in 90 % glycerol in PBS, pH 8.5 to reduce fading of fluorescence. The cells were observed using a Leitz microscope equipped for epifluorescence microscopy and photographed using T-Max 400 film.

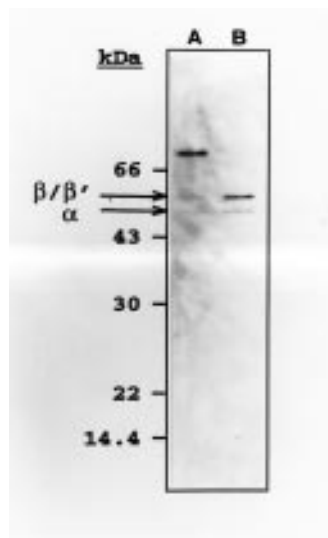
**Cell fractionation and centrosome isolation.** FDCP1 cells were grown in RPMI medium containing 5-10% WEHI-conditioned medium (a source of IL-3), and 10% fetal calf serum. Subcellular fractions consisting of Triton-soluble and insoluble fractions were prepared from cells lysed in 1% (v/v) NP-40. The lysate was centrifuged at 500 x g to pellet nuclei, and the post-nuclear supernatant was spun at 100,000 x g for 60 min. The resulting supernatant is referred to as the Triton-soluble fraction, and the pellet as the Triton-insoluble fraction. Centrosomes were isolated from FDCP1 cells essentially as described for human KE37 T-lymphoblastic cells (13). Cells ( $2 \times 10^9$ ) were treated with  $2 \times 10^{-7}$  M nocodazole and 1  $\mu$ g/ml cytochalasin B for 90 min at 37°C. Cells were harvested by centrifugation at 400 x g for 5 min, washed twice with PBS, and once with 0.1x PBS containing 8% (w/v) sucrose. Cells were lysed in 0.5% (v/v) NP-40, 1mM Tris-HCl, pH 8.0, 0.01 M 2-mercaptoethanol, 0.5 mM

MgCl<sub>2</sub>, 1 mM phenylmethanesulfonyl fluoride, and 10 µg/ml each of aprotinin, leupeptin, and pepstatin A, and centrifuged at 1200 x g for 10 min to pellet nuclei. The post-nuclear supernatant was adjusted to 0.01 M PIPES, pH 7.2 and digested with 600 units of DNase I. The treated supernatant was loaded onto a discontinuous sucrose gradient consisting of 70% (w/v) sucrose at the bottom, followed by 50% and 40% layers made in 0.01 M PIPES, pH 7.2, 0.1% (v/v) Triton X-100, 0.01 M 2-mercaptoethanol. The tubes were centrifuged in a SW28 rotor at 100,000 x g for 60 min and 0.5 ml fractions were collected from the bottom of the tubes. Centrosomes were concentrated by diluting each fraction up to 5 ml with 0.01 M PIPES, pH 7.2 and centrifuged at 50,000 x g in a SW 50.1 rotor for 60 min. The pelleted centrosomes were resuspended in 0.05 ml 0.01 M PIPES pH 6.8 and stored at -70°C. Protein determinations were performed using colloidal gold (Quantigold, Diversified Biotech, Newton Centre, MA) to quantitate submicrogram amounts of protein.

**SDS-PAGE and immunoblotting.** SDG fractions were fractionated by SDS-PAGE (14) and transferred to Immobilon membranes (Millipore, Bedford, MA) using the modification of Otter (15). Gels were transferred in 2x transfer buffer [50 mM Tris-base, 384 mM glycine, 0.01 % (w/v) SDS, 20 % (v/v) methanol] at 80 mA for 60 min, followed by 280 mA for 30 min. After transfer, the membranes were stained with 0.2 % Ponceau S in 1 % acetic acid to visualize the molecular weight standards, and destained with TBS (0.02 M Tris-HCl, pH 7.5, 0.15 M NaCl). The blots were then treated with 0.2% (w/v) glutaraldehyde in PBS for 5 min and washed in TBS. The blots were incubated with 5% (w/v) non-fat dry milk in TBS containing 0.1% Tween-20 (blocking buffer) for 2 hrs at ambient temperature. Antibodies against  $\alpha$ -tubulin, Ca<sup>2+</sup>/CaM kinase II, and centrin were diluted in blocking buffer containing 1 mM CaCl<sub>2</sub> and incubated overnight at 4°C. The blots were rinsed 4 x 30 min each with TBS containing 0.1%

(v/v) Tween 20 and incubated with 1:5000 dilution in blocking buffer of either goat anti-rabbit or anti-mouse IgG-peroxidase conjugate (Amersham) for 1 hr at room temperature. The blots were washed as described above and bound antibodies were visualized by ECL detection (Amersham). The molecular weight markers used for electrophoresis include bovine serum albumin (66 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (22 kDa), and lysozyme (14.4 kDa).

**In vitro phosphorylation.** Endogenous protein phosphorylation was performed in a 10 µl reaction volume containing centrosomes (100 ng protein) in 25 mM HEPES, pH 7.4, 10 mM MgCl<sub>2</sub>, 1 mM EGTA, 1.5 mM Ca<sup>2+</sup>, 30 µg/ml CaM (Sigma, St. Louis, MO) and 5 µCi [ $\gamma$ -<sup>32</sup>P]ATP (3000 Ci/mmol, Amersham) at 37°C for 20 min (12). The reactions were terminated by the addition of 2x Laemmli sample buffer, boiled for 3 min, and analyzed by SDS-PAGE/autoradiography.



**Figure 1.** Enrichment of Ca<sup>2+</sup>/CaM kinase II in the Triton-insoluble fraction of mouse leukemic FDCP1 cells. Aliquots (1 µg each) of the Triton-soluble (A) and -insoluble (B) fractions were separated by SDS-PAGE in 12.5% gels and immunoblotted with anti-Ca<sup>2+</sup>/CaM kinase II antibody (1 µg/ml). The positions of the  $\alpha$  (52 kDa) and  $\beta/\beta'$  (56 kDa) subunits of the kinase are indicated by arrows.

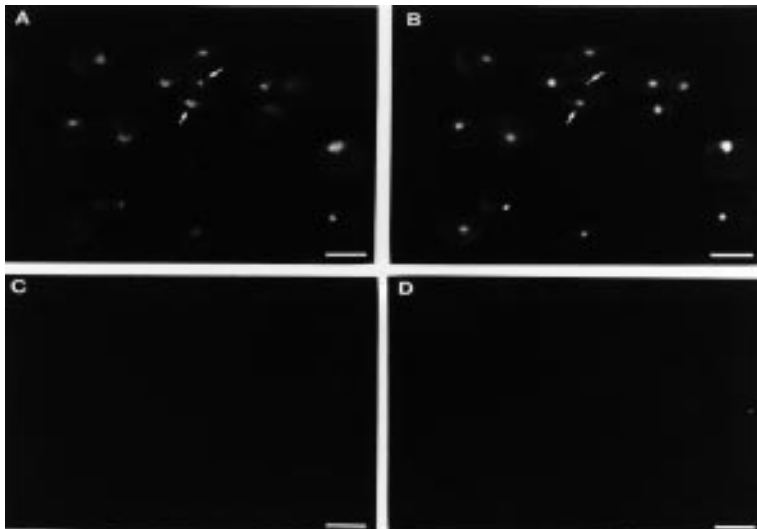
## RESULTS

In order to determine whether Ca<sup>2+</sup>/CaM kinase II is present in FDCP cells and is associated with the cytoskeleton, cells were lysed in 0.5% NP-40 and the post-nuclear supernatant

was centrifuged to obtain Triton-soluble and -insoluble fractions. As shown in Figure 1,  $\text{Ca}^{2+}/\text{CaM}$  kinase II antibody recognizes both the  $\alpha$  (52 kDa) and  $\beta/\beta'$  (56 kDa) subunits in the Triton-soluble (lane A) and -insoluble (lane B) fractions. The Triton-insoluble fraction, which represents 14% of the total protein in the post-nuclear supernatant, contains ~2-fold greater  $\alpha$  and ~4-fold greater  $\beta/\beta'$  subunits than the soluble fraction, which contains 86% of the total protein. Accounting for this difference in protein levels in the Triton-soluble and insoluble fractions, the  $\alpha$  and the  $\beta/\beta'$  subunits are enriched in the detergent-insoluble fraction by factors of 12 and 24 fold, respectively. The antibody also detects a 80 kDa protein in the Triton-soluble fraction (Figure 1, lane A) that has been found in rat aortic vascular smooth muscle cells (16) and in rat heart (17).

We next determined whether  $\text{Ca}^{2+}/\text{CaM}$

kinase II could be detected in centrosomes of FDCP1 cells using immunofluorescence microscopy. Acetone-fixed FDCP1 cells were incubated with affinity-purified rabbit anti- $\text{Ca}^{2+}/\text{CaM}$  kinase II antibody in combination with mouse monoclonal anti-centrin as a marker for centrosomes. As shown in Figure 2, anti- $\text{Ca}^{2+}/\text{CaM}$  kinase II antibody is concentrated in a highly localized, perinuclear region (A), which co-localizes with centrosomes, as revealed by anti-centrin (B). In prometaphase/metaphase cells, where centrosomes occupy opposite poles of the cell, anti- $\text{Ca}^{2+}/\text{CaM}$  kinase II and anti-centrin co-localize in the duplicated centrosomes (Fig. 2 A and B, arrows). Cells incubated with the secondary antibodies alone show no labeling of centrosomes or any other cellular structure (Fig. 2 C and D).



**Figure 2.** Immunofluorescence localization of  $\text{Ca}^{2+}/\text{CaM}$  kinase II in centrosomes. FDCP1 cells were attached to poly-L-lysine glass slides and fixed in acetone. Cells were incubated with affinity-purified rabbit anti- $\text{Ca}^{2+}/\text{CaM}$  kinase II and mouse monoclonal anti-centrin antibodies (A, B) or buffer alone (C, D). The slides were then incubated with goat anti-rabbit IgG (Fab')<sub>2</sub>-FITC and sheep anti-mouse IgG (Fab')<sub>2</sub>-Texas Red. Bar = 10  $\mu\text{m}$ .

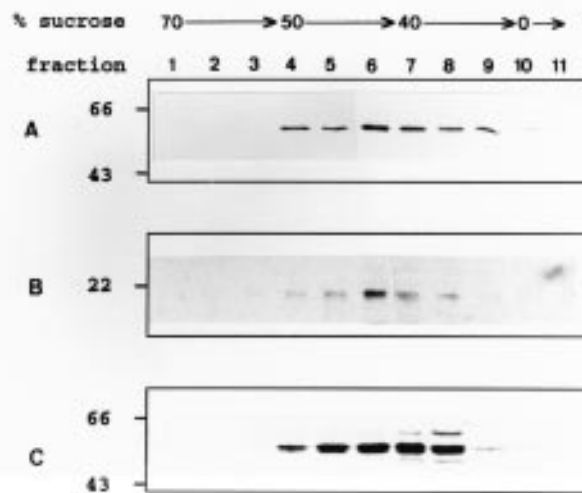
We next determined whether  $\text{Ca}^{2+}/\text{CaM}$  kinase II observed in centrosomes by immunofluorescence could be identified in centrosomes purified from FDCP1 cells. Centrosomes were isolated from FDCP1 cells treated with nocodazole and cytochalasin B using low concentrations of each drug, which are sufficient to depolymerize the microtubule-microfilament

system, but maintain the integrity of centrosomes (13). Cells were lysed in 0.5% NP-40 and the post-nuclear supernatant was fractionated in a sucrose step gradient to isolate fractions enriched in centrosomes. In order to identify fractions containing centrosomes, aliquots of each fraction were subjected to immunoblotting with antibody to centrin, a pericentriolar protein found exclusively in centrosomes (10). These fractions

were also immunoblotted with  $\alpha$ -tubulin antibody to detect tubulin in the centriolar microtubules. As shown in Figure 3, both tubulin at 58 kDa (panel A) and centrin at 20 kDa (panel B) are detected in fractions 4-8 corresponding to 50% sucrose. Fractions 10 and 11 containing soluble protein at the top of the gradient have little detectable centrin, indicating that centrosomes are enriched in the 50% sucrose region of the gradient. Similar results were reported using KE37 cells (13). When centrosomes were isolated in buffer containing 1 mM EDTA (18), centrin was not detected in fractions that would contain centrosomes (50% sucrose). The purity of the centrosome preparation was assessed by immunofluorescence with anti-tubulin antibody, which indicated that all the tubulin was associated with centrosomes (data not shown). Furthermore, the yield of total centrosome protein (2  $\mu$ g, fractions 4-7) from  $2 \times 10^9$  cells represents 0.02 % of total cell protein and corresponds to a

5000-fold purification. The purification attained is comparable to estimates made by other investigators (13, 19).

Immunoblot analysis of the gradient fractions containing both tubulin and centrin (Fig. 3, A and B) with anti- $\text{Ca}^{2+}$ /CaM kinase II antibody reveals the  $\alpha$  and  $\beta/\beta'$  subunits at 52 kDa and 56 kDa, respectively, indicating that  $\text{Ca}^{2+}$ /CaM kinase II co-purifies with centrosome-containing fractions (Fig. 3, panel C). Interestingly, the centrosome fractions contain a greater proportion of  $\alpha$  than  $\beta/\beta'$  subunits (Fig. 3, panel C). Furthermore, immunodetection of these subunits in centrosome fractions corresponds to the antigen detected by immunofluorescence. It is of interest to note that the centrosome fractions do not contain the 80 kDa protein found in the Triton-soluble fraction of total cell lysates, making it unlikely that this is the antigen detected by immunofluorescence.

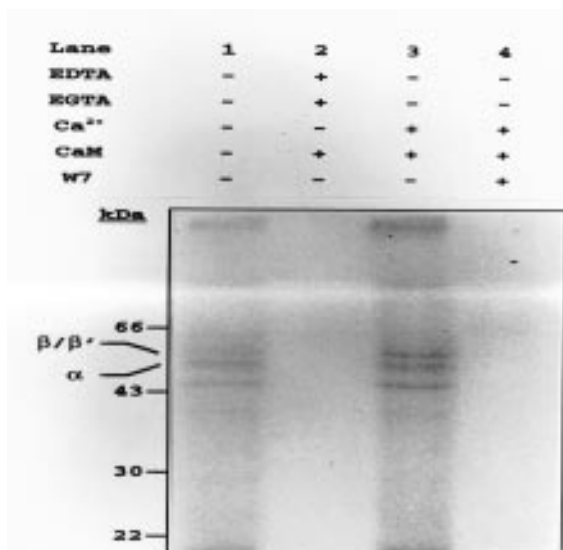


**Figure 3.** Isolation of centrosomes from FDCP1 cells by sucrose density gradient fractionation. A post-nuclear supernatant from FDCP1 cells was fractionated in a sucrose step gradient as described in "Methods". Aliquots (100 ng protein) of each fraction were analyzed by SDS-PAGE in 12.5% gels and immunoblotted with anti- $\alpha$ -tubulin (1:1000 dilution, panel A), anti-centrin (1:2000 dilution, panel B), and anti- $\text{Ca}^{2+}$ /CaM kinase II (1  $\mu$ g/ml, panel C) antibodies.

The centrosome preparation corresponding to fraction 6 that contains the peak of centrin and  $\text{Ca}^{2+}$ /CaM kinase II immunoreactivity was assayed for endogenous  $\text{Ca}^{2+}$ /CaM kinase II enzymatic activity. As shown in Figure 4, basal phosphorylation without added  $\text{Ca}^{2+}$  or CaM is two-fold lower (lane 1) than in the presence of  $\text{Ca}^{2+}$  and CaM (lane 3). The addition of  $\text{Ca}^{2+}$  and

CaM results in phosphorylation of several proteins, the most predominant having molecular masses of 47, 52, 56, 80, and  $>200$  kDa (lane 3). The addition of the CaM antagonist W7, which binds CaM (20), abolishes the phosphorylation of these proteins (lane 4). The inclusion of 1 mM each of EDTA and EGTA, which would reduce  $[\text{Ca}^{2+}]$  to submicromolar amounts while leaving

~8.5 mM  $Mg^{2+}$  in the reaction, abolished endogenous phosphorylation (lane 2). The proteins migrating at 52 and 56 kDa most likely represent the  $\alpha$  and  $\beta/\beta'$  subunits, respectively, of  $Ca^{2+}/CaM$  kinase that are detected by immunoblotting.



**Figure 4.** Protein kinase assay of gradient-purified centrosomes. Autoradiogram of *in vitro* phosphorylation reactions performed as described in "Methods". Code at top shows the composition of each reaction. The concentrations of EDTA and EGTA were 1 mM each, and W7 was 250 mM. The positions the  $\alpha$  and  $\beta/\beta'$  subunits of  $Ca^{2+}/CaM$  kinase II are indicated by the lines. The dried gel was exposed to film for 4 hours.

## DISCUSSION

This study demonstrates that centrosomes purified from FDCP1 cells contain  $Ca^{2+}/CaM$  kinase II. This observation is supported by the *in situ* immunofluorescence localization of  $Ca^{2+}/CaM$  kinase II in centrosomes of FDCP1 cells. Our findings are the first to demonstrate that centrosome-associated  $Ca^{2+}/CaM$  kinase II is activated by  $Ca^{2+}$  and CaM, resulting in the autophosphorylation of the  $\alpha$  and  $\beta/\beta'$  subunits as well as several other unidentified centrosomal proteins. The specificity of the phosphorylation was assessed using the CaM antagonist W7 and the  $Ca^{2+}$ -specific chelator EGTA, both of which inhibited the phosphorylation. Surprisingly, a basal level of phosphorylation without added

$Ca^{2+}$  and CaM was observed in centrosomes isolated in the absence of EDTA, suggesting that sufficient  $Ca^{2+}$  is bound to centrosomes, perhaps via CaM or centrin/caltractin, to activate the kinase. Furthermore, the results presented in this study underscore the importance of previous studies localizing CaM in centrosomes and the spindle apparatus (8).

The centrosome is a microtubule-organizing center, which nucleates polymerization of  $\alpha/\beta$  tubulin to form a microtubular network that is required for cell division, the maintenance of cell shape, and supports intracellular vesicle and organelle transport (21). Microtubule-associated proteins such as MAP-2 and *tau* are required for microtubule assembly and are phosphorylated by  $Ca^{2+}/CaM$  kinase II (22, 23). Our results demonstrating the presence of primarily the  $\alpha$  and some  $\beta/\beta'$  subunits of  $Ca^{2+}/CaM$  kinase II in the Triton-insoluble fraction of FDCP1 cells is consistent with previous studies in neuronal cells describing the association of the enzyme with the cytoskeleton (24). The finding that centrosomes contain primarily the  $\alpha$  isozymic form of  $Ca^{2+}/CaM$  kinase II, while cells contain the  $\beta/\beta'$  subunits as well, raises the possibility that a subset of total intracellular  $\alpha$  polypeptide is targeted to the centrosome. Selective targeting of  $Ca^{2+}/CaM$  kinase II polypeptides has been demonstrated for the  $\delta$  polypeptide, which acquires a nuclear localization signal by alternative splicing (25). However, there is at present a complete lack of information on how intracellular proteins are targeted to the centrosome.

The results presented here have important implications for the role of centrosomal  $Ca^{2+}/CaM$  kinase II. It is now generally accepted that protein phosphorylation is essential for the cell cycle. Several protein kinases, including p34<sup>cdc2</sup> (26), cAMP-dependent protein kinase (27), and  $Ca^{2+}/CaM$  kinase II (28) are active during mitosis and are associated with the centrosome and/or the spindle apparatus. With respect to  $Ca^{2+}/CaM$  kinase II, recent studies suggest that only a transient activation of the kinase is necessary for G<sub>2</sub>-M progression (28).

The expression of a constitutively active,  $\text{Ca}^{2+}$ -independent form of the enzyme in mammalian cells resulted in cell cycle arrest in  $\text{G}_2$ , suggesting that  $\text{G}_2$ -M progression is regulated by a phosphorylation and dephosphorylation cycle. The mechanism by which this signalling pathway affects centrosome function and/or replication is presently not known. However, several studies have shown that epidermal growth factor (EGF) is required for centrosome separation during the cell cycle (29, 30). Extracellular EGF is capable of activating an intracellular  $\text{Ca}^{2+}$ /CaM pathway linking the extracellular milieu with the centrosome replicative cycle and the cell cycle. We are currently studying the activation of  $\text{Ca}^{2+}$ /CaM kinase II in the centrosome by growth factors and the cell cycle-dependent phosphorylation of  $\text{Ca}^{2+}$ /CaM kinase II substrates in the centrosome.

#### ACKNOWLEDGEMENTS

We thank Dr. A. Czernik (Laboratory of Neuroscience, Rockefeller University, New York, NY) for supplying anti- $\text{Ca}^{2+}$ /CaM kinase II. We also thank Dr. J. Salisbury (Department of Biochemistry and Molecular Biology, Mayo Clinic, Rochester, MN) for providing centrin antibody and communicating to us the divalent metal ion-dependent retention of centrin in centrosomes before publication. This research was partially supported by grant # IRG 192 from the American Cancer Society, grant # RO1 CA42520 from NIH, and the University of New Mexico Cancer Center.

#### REFERENCES

- Hanson, P.I., and Schulman, H. Neuronal  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinases. *Annu. Rev. Biochem.* 61: 559-601, 1992.
- Means, A.R., and Dedman, J.R. Calmodulin-an intracellular receptor. *Nature* 285: 73-77, 1980.
- Ohta, Y., Ohba, T., Fukunaga, K., and Miyamoto, E. Serum and growth factors rapidly elicit phosphorylation of the  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinase II in intact quiescent rat 3Y1 cells. *J. Biol. Chem.* 263: 11540-11547, 1988.
- Whitaker, M., and Patel, R. Calcium and cell cycle control. *Development* 108: 525-542, 1990.
- Valentine, M.A., Leprince, C., Genot, E., Meier, K.E., and Clark, E.A. In *Protein kinases in Blood Cell Function*. Huang, C. and Sha'afi, R., eds., CRC Press Inc., London, 1-20, 1992.
- Genot, E.M., Meier, K.E., Licciardi, K.A. et al. Phosphorylation of CD20 in cells from a hairy cell leukemia cell line: Evidence for involvement of calcium/calmodulin-dependent protein kinase II. *J. Immunol.* 151: 71-82, 1993.
- Ohta, Y., Ohba, T., and Miyamoto, E.  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinase II: localization in the interphase nucleus and the mitotic apparatus of mammalian cells. *Proc. Natl. Acad. Sci USA* 87: 5341-5345, 1990.
- Welsh, M.J., Dedman, J.R., Brinkley, B.R., and Means, A.R. Tubulin and calmodulin: effects of microtubule and microfilament inhibitors on localization in the mitotic apparatus. *J. Cell Biol.* 81: 624-634, 1979.
- Dinsmore, J.H., and Sloboda, R.D. Calcium and calmodulin dependent phosphorylation of a 62 kDa protein induces microtubule depolymerization in sea urchin mitotic apparatuses. *Cell* 53: 769-780, 1988.
- Baron, A.T., Greenwood, T.M., Bazinet, C.W., and Salisbury, J.L. Centrin is a component of the pericentriolar lattice. *Biol. Cell* 76: 383-388, 1992.
- Lee, V.D., and Huang, B. Molecular cloning and centrosomal localization of human caltractin. *Proc. Natl. Acad. Sci. (USA)* 90: 11039-11043, 1993.
- Matovicik, L.M., Haimowitz, B., Goldenring, J.R., Czernik, A.J., and Gorelick, F.S. Distribution of calcium/calmodulin-dependent protein kinase II in rat ileal enterocytes. *Am. J. Physiol.* 264: C1029-C1036, 1993.
- Bornens, M., Paintrand, M., Berges, J., Marty, M.C., and Karsenti, E. Structural and chemical characterization of isolated centrosomes. *Cell Motil. Cytoskel.* 8: 238-249, 1987.
- Laemmli, U.K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227: 680-685, 1970.
- Otter, T., King, S.M., and Witman, G. A two-step procedure for efficient electrotransfer of both high-molecular weight (>400,000) and low-molecular weight (<20,000) proteins. *Anal. Biochem.* 162: 370-377, 1987.
- Schworer, C.M., Rothblum L.I., Thekkumkara, T.J., and Singer, H.A. Identification of novel isoforms of the  $\delta$  subunit of  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinase II. *J. Biol. Chem.* 268: 14443-14449, 1993.
- Edman, C.F. and Schulman, H. Identification and characterization of  $\delta_B$ -CaM kinase and  $\delta_C$ -CaM kinase from rat heart, two new multifunctional  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinase isoforms. *Biochem. Biophys. Acta* 1221: 89-101, 1994.
- Sanders, M.A., and Salisbury, J.L. Centrin plays an essential role in microtubule severing during flagellar excision in *Chlamydomonas reinhardtii*. *J. Cell Biol.* 124: 795-805, 1994.
- Mitchison, T.J., and Kirschner, M.W. Isolation of mammalian centrosomes. *Meth. Enzymol.* 134: 261-268, 1986.
- Tanaka, T., Ohmura, T., Yamakado, T., and Hidaka, H. Two types of calcium-dependent protein phosphorylations modulated by calmodulin antagonists: naphthalenesulfonamide derivatives. *Mol. Pharmacol.* 22: 408-415, 1982.
- Vale, R.D. Intracellular transport using microtubule motors. *Ann. Rev. Cell Biol.* 3: 347-378, 1987.
- Matus, A. In *Microtubules*. Hyams, J.S., and Lloyd C.W., eds., Wiley-Liss, New York, 155-166, 1994.
- Goedert, M., Jakes, R., Spillantini, M.G., and Crowther, R.A. In *Microtubules*. Hyams, J.S., and Lloyd C.W., eds., Wiley-Liss, New York, 183-200, 1994.
- Larson, R.E., Espindola, F.S., and Espreafico, E.M. Calmodulin-binding proteins and calcium/calmodulin regulated

- enzyme activities associated with brain actomyosin. *J. Neurochem.* 54: 1288-1294, 1990.
25. Srinivasan, M., Edman, C.F., Schulman, H. Alternative splicing introduces a nuclear localization signal that targets multifunctional CaM kinase to the nucleus. *J. Cell Biol.* 126: 839-852, 1994.
  26. Bailly, E., Pines, J., Hunter, T., and Bornens, M. Cytoplasmic accumulation of cyclin B1 in human cells: association with a detergent-resistant compartment and with the centrosome. *J. Cell Sci.* 101: 529-545, 1992.
  27. Nigg, E.A., Schafer, G., Hilz, H., and Eppenberger, H.M. Cyclic AMP-dependent protein kinase II is associated with the Golgi complex and with centrosomes. *Cell* 41: 1039-1051, 1985.
  28. Planas-Silva, M.D., and Means, A.R. Expression of a constitutive form of calcium/calmodulin dependent protein kinase II leads to cell cycle arrest in G2. *EMBO J.* 11: 507-517, 1992.
  29. Sherline, P. and Mascardo, RN. Epidermal growth factor induces rapid centrosomal separation in HeLa and 3T3 cells. *J. Cell Biol.* 93: 507-511, 1982.
  30. Sherline, P. and Mascardo, RN. Epidermal growth factor-induced centrosomal separation: mechanism and relationship to mitogenesis. *J. Cell Biol.* 95: 316-322, 1982.