Phosphorylation of Centrin during the Cell Cycle and Its Role in Centriole Separation Preceding Centrosome Duplication*

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Once during each cell cycle, mitotic spindle poles arise by separation of newly duplicated centrosomes. We report here the involvement of phosphorylation of the centrosomal protein centrin in this process. We show that centrin is phosphorylated at serine residue 170 during the G2/M phase of the cell cycle. Indirect immunofluorescence staining of HeLa cells using a phosphocentrin-specific antibody reveals intense labeling of mitotic centrioles during prophase and metaphase of the cell division cycle, with diminished staining of anaphase and no staining of telophase and interphase centrosomes. Cultured cells undergo a dramatic increase in centrin phosphorylation following the experimental elevation of PKA activity, suggesting that this kinase can phosphorylate centrin in vivo. Surprisingly, elevated PKA activity also resulted intense phosphocentrin antibody labeling of interphase centrosomes and in the concurrent movement of individual centrioles apart from one another. Taken together, these results suggest that centrin phosphorylation signals the separation of centrosomes at prophase and implicates centrin phosphorylation in centriole separation that normally precedes centrosome duplication.

The centrosome is the major microtubule organizing center of the cell, and as such it determines the number and polarity of cytoplasmic microtubules, as well as the general form of the interphase microtubule array (1). Once in each cell cycle, the centrosome is duplicated to give rise to two centrosomes (spindle poles) that organize the microtubule array of the mitotic spindle. Centrosomes consist of a closely associated pair of centrioles and a pericentriolar matrix that surrounds and connects the individual centrioles to one another and to microtubules. Pericentriolar matrix has the properties of a gel as evidenced by the exclusion of small cytoplasmic particles such as ribosomes and membrane vesicles from the centrosome. At three times during the cell cycle centrioles move apart from one another. The first time occurs immediately following mitosis when the centriole pair transiently splits during or just after telophase. At this time the older centriole remains near the cell center while the younger centriole wanders extensively throughout the cytoplasm before returning to reside near its older partner (2). The second separation of the centriole pair occurs later in G1 phase as cells pass the restriction point and the pair of centrioles become disoriented and slightly separated from one another in the first identifiable event of centrosome duplication (3). The third time centriole pairs move apart from one another occurs as cells enter prophase when newly duplicated centrosomes (each containing a pair of centrioles) separate and migrate to opposite sides of the nucleus where they function as mitotic spindle poles (4). Changes in centrosome function at the time of the G2/M transition are regulated by phosphorylation since centrosomes undergo an increase in protein phosphorylation at this time (5–8). It is likely that specific phosphorylation events also take place at the centrosome at other times during the cell cycle (9). Numerous kinases known to regulate cell cycle progression localize at centrosomes and mitotic spindle poles, including Cdk2 and Cdk4/6, polo-like kinase 1 (PLK1), NIMA-related kinase (Nek2), pEg2, and cAMP-dependent kinase (PKA). Several of the centrosome-specific substrates of these kinases have been identified (for recent reviews, see Refs. 9 and 10). Protein phosphorylation has been implicated in a variety of centrosome functions including centrosome duplication, maturation and separation, microtubule nucleation, and specification of cleavage furrow formation (9–12). Cdk2/cyclin E activity coordinates centrosome duplication with the DNA replication cycle (11–15).

Centrin is a 20-kDa protein of the EF-hand superfamily of calcium-binding proteins that is a component of centrioles themselves and the surrounding pericentriolar matrix (16–18). Gene disruption experiments demonstrate that yeast centrin (Cdc31p) is essential for cell viability (19). Additionally, two distinct centrin mutations have been described that result in either failure of centrosome (spindle pole body) duplication and separation resulting in monopolar spindles and cell cycle arrest, or precocious and inappropriate centrosome separation at the time of cell division resulting in cells with the incorrect number of centrioles/basal bodies (20–22). Three separate human centrin genes that encode and express centrin have been identified (23–25). A conserved carboxyl-terminal region of centrins from diverse species includes a consensus motif for protein phosphorylation that is typical for serine/threonine kinases (26).

Centrin phosphorylation in lower eukaryotes has been shown to correlate with extension of centrin-containing fibers associated with centrioles/basal bodies (27,28). More recently, aberrant centrin phosphorylation has been demonstrated in human breast tumors that have amplified centrosomes con-
taining supernumerary centrioles and/or excess pericentriolar material (29). The objective of this study was to investigate the cell cycle-dependent phosphorylation of centrin and to characterize the specific site of phosphorylation on the protein. Using an antibody that is specific for phosphorylated centrin and standard biochemical methods, we demonstrate that in cultured vertebrate cells, centrin is phosphorylated near its carboxyl terminus at serine residue 170 early in mitosis when the newly duplicated centrosomes separate to give rise to the mitotic spindle poles. The spindle pole localization of phosphocentrin remains high until metaphase and then diminishes to basal levels by telophase. The timing of centrin phosphorylation suggests that phosphorylation of centrin may initiate the separation of duplicated centrosomes in preparation for mitotic spindle formation. Experimental elevation of protein kinase A (PKA) activity in interphase cells also results in the phosphorylation of centrin at serine residue 170 and the concomitant movement of centrioles away from one another in a similar manner to the transient separation of the pair of centrioles that normally occurs preceding centrosome duplication which begins at about the time of the G2/S transition. These observations suggest that centrin phosphorylation plays a role in mitotic spindle pole and centriole separation at key stages of the cell cycle.

EXPERIMENTAL PROCEDURES

Reagents—Rat brain protein kinase C was obtained from Calbiochem, cyclin-dependent protein kinase 1 (Cdc2 kinase) from Boehringer Mannheim, cyclin-dependent protein kinase 2 (Cdk1) from Boehringer Mannheim, and recombinant human CETN2p expressed in E. coli was purified using standard biochemical methods, we demonstrate that in cultured vertebrate cells, centrin is phosphorylated near its carboxyl terminus at serine residue 170 early in mitosis when the newly duplicated centrosomes separate to give rise to the mitotic spindle poles. The spindle pole localization of phosphocentrin remains high until metaphase and then diminishes to basal levels by telophase. The timing of centrin phosphorylation suggests that phosphorylation of centrin may initiate the separation of duplicated centrosomes in preparation for mitotic spindle formation. Experimental elevation of protein kinase A (PKA) activity in interphase cells also results in the phosphorylation of centrin at serine residue 170 and the concomitant movement of centrioles away from one another in a similar manner to the transient separation of the pair of centrioles that normally occurs preceding centrosome duplication which begins at about the time of the G2/S transition. These observations suggest that centrin phosphorylation plays a role in mitotic spindle pole and centriole separation at key stages of the cell cycle.

EXPERIMENTAL PROCEDURES

Reagents—Rat brain protein kinase C was obtained from Calbiochem, cyclin-dependent protein kinase 1 (Cdc2 kinase) was obtained from Upstate Biotechnology Inc., and [γ-32P]ATP was obtained from PerkinElmer Life Sciences. Sulforhodamine B (SRB) was obtained from Sigma and used for cell counts. HCT-12 (Table I) was conjugated to keyhole limpet hemocyanin and used to immunize female New Zealand White rabbits. Antisera from the third and fourth bleeds were used for the experiments described in this work. An IgG fraction was prepared using a protein-A-Superose column (Amersham Pharmacia Biotech) and the methods supplied by the manufacturer. The IgG fraction was pre-cleared with a polyclonal rabbit antibody (anti-histone) and applied to a nitrocellulose membrane. The membrane was incubated with 10 µg of phosphorylated centrin and 0.25 units of purified PKC from rat brain for 1 h. The IgG fraction was purified by SDS-PAGE and transferred to Immobilon-P membrane (Millipore) according to the method of Towbin and colleagues (37). The region of the membrane containing phosphocentrin was localized by autoradiography, and subjected to trypsin proteolysis and phosphoamino acid analysis, as described by Boyle and co-workers (37).

Antiserum Production and Affinity Purification of aHCT-P Antibodies—The synthetic HCT peptide (Table I) was conjugated to keyhole limpet hemocyanin to form a protein-A-Superose column (Amersham Pharmacia Biotech) and the methods supplied by the manufacturer. The IgG fraction was pre-cleared with a polyclonal rabbit antibody (anti-histone) and applied to a nitrocellulose membrane. The membrane was incubated with 10 µg of phosphorylated centrin and 0.25 units of purified PKC from rat brain for 1 h. The IgG fraction was purified by SDS-PAGE and transferred to Immobilon-P membrane (Millipore) according to the method of Towbin and colleagues (37). The region of the membrane containing phosphocentrin was localized by autoradiography, and subjected to trypsin proteolysis and phosphoamino acid analysis, as described by Boyle and co-workers (37).

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motif (KK carboxyl termini (Table I) that includes a consensus amino acid sequence of an equal molar mixture of recombinant centrin, citrate lyase, l-phenylalanine hydroxylase, and phosphorylase kinase. 4) Specificity of αHCT-P IgG for centrin phosphorylated by PKA versus PKC or Cdc2 kinase was determined by autoradiography and Western blotting following in vitro phosphorylation of centrin by each of the respective kinases.

Immunoprecipitation and Western Analysis—Cell lysates were prepared for immunoprecipitation and Western blot analysis as described previously (23). For the experiments described here, extracts were immunoprecipitated using polyclonal serum 26/14-1 that recognizes centrin regardless of its phosphorylation state or the preimmune serum from the same rabbit. For Western blot analysis, centrin was resolved by SDS-PAGE, transferred to Immobilon-P (polyvinylidene difluoride) membrane (Millipore), and fixed with 0.2% glutaraldehyde followed by standard procedures according to Towbin and co-workers (59) and probed using either monoclonal anti-centrin 20H5 or polyclonal centrin regardless of phosphorylation state or αHCT-P IgG.

Immunofluorescence—HeLa cells grown on glass coverslips were fixed in cold methanol (−20 °C) for 10 min and processed for indirect immunofluorescence microscopy as described previously (23) using α-HCT-P IgG or polyclonal anti-centrin 26/14-1.

RESULTS

Analysis of Centrin Phosphorylation Status during the Cell Cycle—Immunoprecipitation and Western blot analysis of centrin (20 kDa) from untreated cells is shown in Fig. 1A. Cell cycle analysis and the corresponding phosphorylation status of centrin in untreated (control asynchronously cycling) cells or cells subject to cell cycle arrest by thymidine (S phase) or nocodazole treatment (G2/M phase) are shown in Fig. 1, B and C, respectively. Incorporation of 32P into centrin was highest in cultures arrested in S phase by thymidine treatment, while cultures arrested in G2 phase by thymidine treatment showed the lowest levels of incorporation and control (cycling) cultures showed an intermediate level of 32P incorporation. These results suggest that centrin phosphorylation is maximum during mitosis.

Phosphorylation of Centrin by PKA in Vitro—Centrins from a variety of species share a highly conserved sequence at their carboxyl terminus (Table I) that includes a consensus amino acid motif (KXXS/X) for phosphorylation by PKA (38). Bacterially expressed centrin and a synthetic peptide, HCT-12, corresponding to the carboxyl-terminal 12 amino acids of CETN2 (Table I), are readily phosphorylated by PKA in vitro (Fig. 2). Tryptic digestion and two-dimensional analysis of the phosphorylated products reveals a single negatively charged major and a neutral minor 32P-labeled tryptic peptide for both the recombinant protein and the synthetic peptide (Fig. 2, B and C, respectively). The two 32P-labeled peptide fragments comigrate when tryptic digests of centrin and the synthetic peptide are first mixed and subsequently run together by two-dimensional peptide analysis (Fig. 2D). Phosphoamino acid analysis demonstrates that serine is the 32P-labeled residue for both the major and minor peptide fragments (Fig. 2, E and F). Comparison of migration of tryptic peptides from centrin with the predicted pattern based on the computer program of Hunter (37) and the fact that there is only one potential serine phosphorylation site in the synthetic HCT peptide, allowed the unequivocal identification of serine residue 170 in the carboxyl-terminal sequence KKTS\(^2\)Y as the site for in vitro phosphorylation of centrin by PKA. The major negatively charged phosphopeptide (TS\(^2\)LY) represents the carboxyl terminus (residues 169–172) of centrin and the minor neutral peptide fragment (KTSS\(^2\)LY) represents an alternative partial tryptic product (residues 168–172) resulting from the less efficient trypsin cleavage activity between adjacent lysine residues (37).

Centrin Phosphorylation during the cell cycle. A, centrin (20 kDa) from cultured QT6 cells is specifically identified by Western blot using monoclonal anti-centrin 20H5 following immunoprecipitation with immune serum anti-centrin 26/14-1 (lane i) but not preimmune serum (lane p) from the same rabbit. Molecular mass markers (in kDa): 200, 116, 97, 66, 45, 31, 21, 14 (top to bottom). B, flow cytometry of cultured cells that were either untreated (control asynchronously cycling), or arrested in the cell cycle in S-phase (thymidine) or G2/M (nocodazole). Bars indicate 2C and 4C DNA content. C, incorporation of 32PO\(_4\) into centrin in cultured cells treated as above for flow cytometry: Western, blot of immunoprecipitated centrin; α2P, autoradiograph of the same lanes. Cont, control cycling cells; thy, thymidine; noc, nocodazole.
TABLE I

Carboxy-terminal amino acid sequences for centrin

| Species     | Accession No. | Sequence                      |
|-------------|---------------|-------------------------------|
| Human 1     | U03270        | HCT-P X69706f                 |
| Human 2     | X72964        | HCT-P X69706f                 |
| Human 3     | Y12473        | HCT-P X69706f                 |
| Xenopus     | U37538        | HCT-P X69706f                 |
| Chlamydomonas | X57973     | HCT-P X69706f                 |

B. PKA phosphorylation consensus motif

KKXS*X consensus (26)
KKTS*LY human CETN1 and CETN2

C. Synthetic peptides

HCT-12
HCT-P

cEFLRIMKKTSLY
cEFLRIMKKTSLY

FIG. 2. Two-dimensional tryptic peptide maps and phosphoamino acid determinations for phosphocentrin and synthetic HCT-P peptide. Centrin and the HCT peptide were phosphorylated in vitro using PKA and [γ-32P]ATP. A. A phosphocentrin was resolved by SDS-PAGE and the HCT-P was resolved by SDS-Tricine PAGE (not shown). Tryptic phosphopeptide maps for centrin (B), HCT-P (C), and a mixture of the two (D) are shown; arrows indicate the minor neutral and major positively charged peptides, asterisk (*) indicates the origin, and minus and plus (− and +) indicate polarity for electrophoresis. E and F, autoradiographs of chromatograms for phosphoamino acid determinations of the major and minor peptide spots, respectively. The positions of ninhydrin-stained phosphoamino acid standards (phosphoserine, phosphothreonine, and phosphotyrosine), the origin and free 32PO4 are indicated. Synthetic HCT-P and the minor neutral and major negative tryptic products are indicated.

centrin, followed by in vitro incubation under complete reaction conditions with PKA and [γ-32P]ATP, and subsequent analysis by SDS-PAGE, autoradiography, and Western blotting (Fig. 3C). In this experiment the affinity purified aHCT-P IgG specifically and exclusively recognized phosphocentrin and no other PKA-phosphorylated proteins (Fig. 3C, Western (w)), even when present at equivalent levels and having significant 32P incorporation (Fig. 3C, autoradiograph (a)). A second control experiment was performed in which centrin was phosphorylated by one of three distinct protein kinases: PKA, PKC, or p44/42 MAP kinase (Cdc2) and subsequently analyzed by Western blotting with monoclonal 20H5 or aHCT-P IgG (Fig. 3D). Centrin is phosphorylated by all three kinases in vitro (Fig. 3D, 32P) and analysis by phosphoprotein mapping demonstrated that each kinase tested phosphorylated distinct sites on centrin (not shown). Only PKA phosphorylation resulted in gel mobility retardation of centrin and only the PKA-phosphorylated form of centrin reacted with the affinity purified aHCT-P IgG (Fig. 3D, aHCT-P). Taken together, these results demonstrate that affinity purified aHCT-P IgG specifically recognized centrin only when it was phosphorylated on the extreme carboxy-terminal amino acid serine located at residue 170. In addition, this antibody recognized no other PKA-phosphorylated proteins or centrin itself when it was exclusively phosphorylated at sites other than serine residue 170.

Phosphorylated Centrin Localizes at Mitotic Spindle Poles in

HeLa—HeLa cells grown on glass coverslips were processed for indirect immunofluorescence using affinity purified aHCT-P IgG. When fields of subconfluent cells were observed by indirect immunofluorescence microscopy a vast majority of the cells showed no apparent labeling above background when stained with aHCT-P IgG (Figs. 4C and 5, A and B). Remarkably, however, dividing cells present throughout the microscope field showed clear and intense staining of their mitotic spindle poles (Figs. 4, C-F, and 5, A and B). The specificity of immunostaining of mitotic spindle poles by aHCT-P IgG was evaluated following competition of the antibody preparation with the peptides HCT-P and HCT. The affinity purified aHCT-P IgG was used at a constant and known dilution (1.5 μg/ml IgG), preincubated with known amounts of the phosphorylated peptide HCT-P or the nonphosphorylated peptide HCT, and subsequently used for indirect immunofluorescence labeling of HeLa (Fig. 4, A-F). Preincubation of aHCT-P IgG with HCT-P peptide at a molar ratio of 1:1 (antibody:peptide, calculated based on antigen-binding equivalents on the IgG) completely eliminated subsequent staining of prophase and metaphase spindle poles (Fig. 4A), while preincubation at a molar ratio of 1:0.1 resulted in a reduction but not complete elimination of subsequent staining (Fig. 4B), and aHCT-P IgG that was not preincubated with any peptide resulted in bright staining of spindle poles (Fig. 4C) as described above. However, preincubation of the aHCT-P IgG with the nonphosphorylated peptide HCT showed reduction in staining of mitotic spindle poles at 1:0.1 (Fig. 4F), 1:1 (Fig. 4E), or even 1:1000 (Fig. 4D) molar ratios of antibody:peptide (antigen-binding equivalents on the
**Fig. 4.** Indirect immunofluorescence micrographs of HeLa cells stained with αHCT-P IgG and FITC-conjugated secondary antibody following preincubation of the primary antibody with competing peptides. C, control without competing peptide reveals αHCT-P IgG staining of metaphase spindle poles but not interphase centrosomes. B, preabsorption of αHCT-P IgG with 0.1:1-fold excess competing HCT-P peptide prior to staining results in diminution of specific label, while preabsorption with HCT-P at 1:1 stoichiometry of competing peptide (A) completely abolishes label. Competition with 0.1:1-, 1:1-, and 1000-fold excess of the nonphosphorylated peptide HCT results in no diminution of label. Note the bright staining of poles in the metaphase (m) spindle and lack of staining of centrosomes in interphase cells (i) and at the poles of the telophase (t) stage cell in E.

IgG:HCT). These results demonstrate that spindle pole labeling by αHCT-P IgG is exquisitely specific for the phosphorylated carboxyl-terminal serine residue 170 of centrin.

Careful analysis of a large number of mitotic cells (Fig. 5, A and B) shows that prophase and metaphase spindle poles stained most intensely with αHCT-P antibodies. Staining of anaphase spindle poles was present, albeit at diminished levels, and telophase spindle poles (see Fig. 4E) failed to stain at all. That centrin is present in interphase centrosomes and at the spindle poles during all stages of mitosis is demonstrated by staining with the polyclonal serum 26/14-1 (Fig. 5C). We interpret these indirect immunofluorescence studies to demonstrate that HeLa centrin becomes phosphorylated at serine residue 170 in a cell cycle-specific manner making the carboxyl-terminal domain recognizable by αHCT-P IgG. Since staining by αHCT-P IgG is most intense during prophase and metaphase, the phosphorylation state of centrin is likely to be highest at these times.

**Activation of PKA Stimulates in Vivo Phosphorylation of Centrin and Centriole Separation in Interphase Cells**—The observations presented above do not necessarily demonstrate that PKA is the kinase responsible for phosphorylation of centrin in vivo, nor do they eliminate the possibility that other kinases may also phosphorylate distinct sites on centrin either at the time of mitosis or at other times during the cell cycle. In order to address the former question, cell cultures were incubated under control conditions or under treatment conditions that elevate endogenous cAMP levels to activate PKA. Cultures were allowed to incorporate 32P-O4- for 4 h prior to treatment for 2 min with the membrane permeable cAMP analog (100 μM Bt2cAMP) that selectively activates PKA, with or without concomitant treatment with the cAMP phosphodiesterase inhibitor IBMX (1 mM). Cells were lysed, and centrin was immunoprecipitated using 26/14, resolved by SDS-PAGE, and subsequently analyzed by Western blotting and autoradiography for centrin phosphorylation. Control cells show low but detectable levels of centrin phosphorylation (Fig. 6), while cells treated with Bt2cAMP alone or Bt2cAMP and IBMX show substantially increased centrin phosphorylation. The centrin Western blot appears as a closely spaced double band, with the more slowly migrating band corresponding to the major phosphorylated product. A third band also appears following treatment with both Bt2c- and IBMX and represents a minor hyperphosphorylated product migrating higher in the gel. This experiment demonstrates that treatment of living cells with cAMP analogs to activate PKA results in an increase in the phosphorylation of centrin. Immunofluorescence staining using αHCT-P IgG of HeLa cells similarly treated to elevate PKA activity (in the presence of calyculin A, an inhibitor of protein phosphatases 1 and 2A) demonstrates staining of mitotic cells and shows discrete αHCT-P IgG staining of their spindle poles as seen before. Remarkably however, in individual interphase cells treated to elevate PKA activity, centrosomes acquire the phosphocentrin epitope necessary for labeling with αHCT-P IgG (Fig. 7, B and C). In addition, the αHCT-P IgG-labeled interphase centrosomes appear as two spots separated by 2–4 μm, suggesting interphase centrosomes have separated into two discrete entities (Fig. 7, B and C). Typically, one of the spots is brighter than the other. Control cells (not treated with cAMP analogs) labeled with the polyclonal anticentrin serum 26/14-1 that recognizes centrin regardless of phosphorylation state show a single staining spot of a non-separated centrosome (Fig. 7D). Centri-
mitotic spindle assembly occur. Furthermore, cultured cells showed a dramatic and rapid increase in centrin phosphorylation following experimental treatment (Bt$_2$CAMP and IBMX) that elevates PKA activity, suggesting that this kinase can phosphorylate centrin \textit{in vivo}. Artificial elevation of \textit{in vivo} cAMP levels resulted in phosphocentrin-specific antibody staining of centrosomes in interphase HeLa cells in addition to the mitotic spindle pole label seen without drug treatment. Surprisingly, elevated cAMP also resulted in precocious separation of centrosomes into two distinct spots that we interpret to represent the displacement of individual centrioles a short distance from one another.

Taken together these results indicate human centrin is phosphorylated near its the carboxy terminus at serine residue 170 early in mitosis by PKA or another protein kinase with a substrate specificity similar to PKA. Observations which favor the \textit{in vivo} phosphorylation of centrin by PKA include the following: 1) a conserved PKA consensus phosphorylation motif in the carboxy terminus of centrin; 2) the \textit{in vitro} phosphorylation of centrin by PKA at this site; and 3) the stimulation of centrin phosphorylation \textit{in vivo} by cell permeable cAMP analogs and treatments that reduce phosphodiesterase and phosphatase activity (IBMX and calyculin A, respectively). Moreover, PKA is localized at the centrosome in vertebrate cells, including HeLa (39, 40) through interaction of its regulatory subunit (RII) and the protein kinase A anchoring coiled-coil domains of AKAP450 and the centrosomal structural protein pericentrin (41–43). Tethering of PKA to protein kinase A anchoring proteins is thought to target the enzyme to the proximity of relevant substrates, thereby conveying spatial specificity to cAMP/PKA signaling.

Numerous studies demonstrate that cellular levels of cAMP in HeLa and other cultured cells are maximal in G$_1$ of the cell cycle and minimal in G$_2$/M (44–46). High levels of cAMP seen during G$_1$ phase correspond to the time of transient centriole separation following mitosis. However, the period when centrin phosphorylation levels appear highest (G$_2$/M) corresponds to the time when cellular cAMP levels are at their lowest. Furthermore, cAMP has been shown to delay G$_2$ progression, inhibit cell proliferation, and negatively regulate mitotic Cdc2 kinase activity (47–49). These observations confound a possible role for PKA-mediated phosphorylation of centrin at the onset of mitosis. It is possible that PKA acting at the centrosome is regulated through local changes in the cAMP pool that are not reflected in the overall cellular levels of cAMP. This could be accomplished through the activation of adenylyl cyclase at times when the cAMP pool is low as is seen in G$_2$/M (44), and/or through competition between PKA and protein kinases (50, 51) that are tethered at the centrosome near their substrates. Alternatively, a kinase(s) other than or in addition to PKA may phosphorylate centrin at G$_2$/M.

As indicated earlier, ~20 kinases have been localized at the centrosome, some only transiently and others throughout the cell cycle (9). As far as kinases are concerned the centrosome is a very crowded place. The cyclin-dependent kinases Cdk2 and Cdk4/6 are of particular interest because of their role in regulation of cell cycle progression and because centrosome duplication has been demonstrated to be dependent on Cdk2/cyclin E or cyclin A activity in \textit{Xenopus} cell-free extracts and somatic cells, respectively (3, 12, 15, 52). Our studies show that centrin can indeed be phosphorylated by Cdk in \textit{vitro}, however, at a site that is distinct from serine 170 recognized by the phosphocentrin-specific antibody described in this study.

The functional consequences of centrin phosphorylation are not known. Genetic studies in lower eukaryotes suggest centrin plays an essential role in centrosome duplication and/or sepa-

**FIG. 7.** Stimulation of PKA results in extraordinary phosphorylation of centrosomes and in centriole separation in interphase HeLa cells. A, indirect immunofluorescence of HeLa cells stained with aHCT-P IgG following control (Me$_2$SO) treatment. As before, metaphase spindle poles stain while interphase cells show no specific label. B and C, treatment with Bt$_2$CAMP, IBMX, and calyculin A for 30 min to elevate PKA activity results in aHCT-P IgG staining of interphase centrosomes as well as mitotic spindle poles. Interphase centrosomes of treated cells show two stained spots (paired arrows) demonstrating that activation of PKA results in separation of centrioles from one another. D and E, control cells stained with 26/14-1 show typical staining of interphase centrosomes. Bar in A for A, B, and D, and in E for C and E, 20 µm.
Bipolar mitotic spindle. Duplicated centrosomes separate from one another to form the troponin C, which regulates the Ca^{2+} in a manner similar to that of another member of the calmodulin superfamily, troponin C, which regulates the Ca^{2+}—dependent contraction of skeletal muscle. In this regard, calcium binding by centrin stimulates centriole-associated fiber contraction while calcium phosphorylation and Ca^{2+} release are associated with fiber extension (27, 28, 58). Thus, the observations reported in the present work suggest that centrin phosphorylation may play a role in solution or extension of the gel-like pericentriolar matrix at during G1 phase when centriole pairs undergo disorientation initiating centrosome duplication and at G2/M when newly duplicated centrosomes separate from one another to form the bipolar mitotic spindle.

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