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Cell cycle-dependent localization of casein kinase I to mitotic spindles

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ABSTRACT **Casein kinase I (CKI) is a class of protein kinases ubiquitous to all eukaryotic cells. Recently, cDNA clones encoding several bovine CKI isoforms have been sequenced that show high sequence identity to the HRR25 gene product of the budding yeast *Saccharomyces cerevisiae*; HRR25 is required for normal cellular growth, nuclear segregation, DNA repair, and meiosis. We have raised polyclonal antibodies to a human erythroid 34-kDa CKI and have sequenced a portion of this kinase. The amino acid sequence identifies the CKI as the α-CKI isoform, which is 62% identical to the HRR25 protein kinase. By use of immunofluorescence, the α-CKI has been localized to vesicular cytosolic structures and to the centrosome in interphase cells. As cells progress into mitosis, centrospheric staining increases and, in mitosis, α-CKI associates with kinetochore fibers. This localization suggests that α-CKI, like HRR25, plays a role in the segregation of chromosomes during mitosis and may be cell cycle-regulated both in humans and in yeast.**

Casein kinase I (CKI) activity is ubiquitous in eukaryotes from human to yeast (1). CKI is an unusual protein kinase in that it has been isolated in active form from the cytosol, membranes, and nuclei (1–3). Generally, CKI from the cytosol or membranes of mammalian cells has an apparent molecular mass of 30–37 kDa (1–3). When CKI has been purified from nuclei, a larger range has been reported, 25–55 kDa (1). Indeed, a 46-kDa casein kinase from yeast is conserved in vertebrates and is located in the nucleus in mouse cells (4). CKI from human erythrocytes is a monomer when purified and has a molecular size of 34–36 kDa, depending upon the phosphorylation state of the enzyme (1, 2, 5).

CKI phosphorylates the cytoskeletal proteins myosin, ankyrin, troponin, spectrin, and protein 4.1; neural filaments; neural cellular adhesion molecules; RNA polymerases I and II; translation initiation factors 4B, 4E, and 5; tRNA synthetases; simian virus 40 large T antigen; the insulin receptor; the regulatory subunit (phosphatase inhibitor 2) of protein phosphatase 1; the erythrocyte anion transporter; and metabolic enzymes, including glycogen synthase (reviewed in ref. 1). Some of these substrates undergo defined functional changes when phosphorylated by CKI. For example, *in vivo*, protein phosphatase 1 (phosphatase inhibitor 2) appears to be phosphorylated by CKI and this inhibits the phosphatase activity (6).

Glycogen synthase, when phosphorylated by cAMP-dependent protein kinase and then by CKI, is potently inhibited (7). This suggests that CKI phosphorylates substrates synergistically with other protein kinases. In other words, there is a sequence or hierarchy of phosphorylation. Indeed, Roach and others (8–12) have shown that substrates for CKI contain the sequence similar to -Asp_4-Xaa_2-Ser- or -Ser(*P*)-Xaa_2-Ser-, where good substrates have negative residues upstream of the phosphorylated residue, and the best substrates have a phosphorylated residue upstream. Other substrates that are hierarchically phosphorylated by CKI include the neural cell adhesion molecule and the phosphatase 1 inhibitory subunit (6). It is likely that protein kinases other than cAMP-dependent protein kinase also synergistically phosphorylate proteins that then become CKI substrates.

The erythrocyte CKI is regulated by interactions with both integral proteins and phosphatidylinositol 4,5-bisphosphate (PIP_2) in membranes (2, 3). In erythrocytes, the assembly of CKI onto membranes is regulated by PIP_2. The protein kinase activity is also inhibited by PIP_2, and this occurs over a small change in the total PIP_2 content in the membranes (3). This unusual pattern of inhibition may occur as a result of the sequestration of membrane PIP_2 into "pools" and may provide a simple mechanism for regulation of membrane-associated 34-kDa CKI by PIP_2. Cells and membranes are known to sequester the inositolphospholipids in metabolic pools that have different susceptibilities to phospholipase C or to the phosphomonoesterases that turn over phosphate residues on the 4- or 5-hydroxyl. Pooling of PIP_2 may be a result of associations with membrane proteins or with membrane skeletal proteins (13–17). An increase in membrane PIP_2 may saturate the sequestered PIP_2 pool, increasing the "free" PIP_2, which then modulates a number of enzymes, including the 34-kDa CKI activity (18–21). Such a mechanism could regulate both CKI activity and association with membranes but would require only a small change in total membrane PIP_2 content.

Recently, several cDNA clones for CKIs have been isolated and sequenced (22–24). These sequences identify the CKIs as a distinct family of protein kinases that have low identity with other protein kinases. For example, α-CKI has <24% identity with casein kinase II, calcium/calmodulin-dependent protein kinase II, or cAMP-dependent protein kinase catalytic subunit (22–24). However, within the family, the CKIs are quite homologous: 50% identical or greater. The bovine α-CKI is 62% identical to the HRR25 protein kinase in *Saccharomyces cerevisiae* (22, 23). The HRR25 protein kinase was originally discovered as a gene involved in DNA repair (23). In *S. cerevisiae*, deletion of the HRR25 gene results in a phenotype in which the yeast cannot enter meiosis, and during mitosis a fraction of cells are defective in nuclear segregation (23). Two other protein kinases from yeast have been cloned (YCK 1 and 2) that are homologous to the bovine CKIs; deletion of either of these CKIs results in no phenotypic change, but when both are deleted, the cells are not viable (24).

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Abbreviations: BSA, bovine serum albumin; CKI, casein kinase I; ER, endoplasmic reticulum; PIP_2, phosphatidylinositol 4,5-bisphosphate.

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MATERIALS AND METHODS

Preparation of Antibodies to 34-kDa CKI and Immunoprecipitation. Human 34-kDa CKI was purified from erythrocyte cytosol and used for antibody production and amino acid sequence determination (2, 3). The purified CKI (2 mg) was subjected to preparative SDS/7–15% PAGE and stained with Coomassie blue, and the 34-kDa CKI was electroeluted (25). The electroeluted CKI was coupled to 0.5 mg of keyhole limpet hemocyanin (KLH) by using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (Pierce) (25). Rabbits were immunized twice with CKI-KLH and then boosted intravenously with 100 μg of electroeluted CKI that had been dialyzed into phosphate-buffered saline (PBS). Sepharose CL-4B was activated by CNBr activation, and the activated beads were combined with electroeluted 34-kDa CKI, as described (25). Antibodies specific for CKI were purified using the CKI-Sepharose column; antiserum was applied to the column, which was then washed with PBS containing 0.5 M NaCl, and the antibodies were eluted with 0.1 M glycine at pH 3.0 (25). SDS/PAGE of the affinity-purified antibodies gave only two bands, and these corresponded to the size of IgG heavy and light chains. For immunoprecipitation, anti-34-kDa CKI antibodies were combined with protein samples containing 34-kDa CKI and incubated for 30 min at 4°C. Then 0.5 mg of Dynabeads M-280 (Dynal, Great Neck, NY) coated with sheep anti-rabbit IgG [prewashed with PBS/0.1% bovine serum albumin (BSA)] was added to each sample and incubated at 4°C for 1 hr. The samples were diluted 4-fold with PBS/0.1% BSA, and the beads were collected using a magnetic particle concentrator and then washed three times with PBS/0.1% BSA.

Protein Kinase Assays. CKI was assayed using α-casein as the substrate (2, 3). Briefly, protein phosphorylation was started by addition of 50 mM Tris Cl, pH 7.5/5 mM $MgCl_2$/50 μM [γ-^{32}P]ATP (30 Ci/mol; 1 Ci = 37 GBq). After 5 min, reactions were quenched with concentrated Laemmli sample buffer. Proteins were separated by SDS/7–15% PAGE, and the bands corresponding to casein were excised for scintillation counting.

Amino Acid Sequencing. Human 34-kDa CKI, purified as above, was electroeluted from a preparative SDS/polyacrylamide gel; the SDS was extracted by −20°C ethanol precipitation of the CKI and the ethanol was removed by high vacuum. The 34-kDa CKI was dissolved in 8 M urea, diluted 2.7-fold with 100 mM Tris Cl (pH 9) containing *Achromobacter* lysine-specific protease, and digested overnight at 37°C (26). The resulting peptides were purified by reverse-phase HPLC on a C_4 column. The purified peptides were sequenced by automated Edman degradation.

Cell Culture. Chinese hamster ovary (CHO) and B82 mouse fibroblast cells were grown in Ham's F-10 and Dulbecco's modified Eagle's medium, respectively, with 10% fetal bovine serum. For immunofluorescence, cells were grown on glass coverslips and fixed and stained for immunofluorescence at 50–70% confluence.

Western Blotting. Affinity-purified antibodies were used for blotting and immunofluorescence. For blotting, cells were lifted from culture dishes with PBS/2 mM EDTA and lysed in hot SDS sample buffer. Cold oocytes and flies were lysed in hot SDS sample buffer and Dounce homogenized. Yeast cells, strain Σ1278b, were lysed with a French pressure cell (total lysate), and membranes were prepared as described (27).

Immunofluorescence. For staining intact cells, coverslips were washed once in PBS at 37°C and fixed in −20°C methanol for 30 min. To destabilize microtubules, cells were treated with nocodazole (10 μg/ml) in medium and then fixed as above. For staining permeabilized cells in microtubule-stabilizing conditions, coverslips were washed at 37°C in PEM (0.1 M Pipes, pH 6.9/1 mM $MgSO_4$/2 mM EGTA) and then in PEM/0.2% Triton X-100/2 mM dithiothreitol (28–30), then fixed and stained. Fixed cells were washed in PBS (20°C) and blocked in PBS/3% BSA/0.1% Tween 20 for 1–24 hr. Coverslips were then incubated with affinity-purified CKI antibody (8 μg/ml) in PBS/3% BSA for 35 min at 37°C; a 1:1000 dilution of β-tubulin antibody (Amersham) was added and incubated for a further 35 min at 37°C. As a control, CKI antibody was preincubated with CKI-Sepharose or pure denatured CKI. Coverslips were washed five times (10 min each) in PBS/3% BSA and then incubated for 1 hr at 20°C with a fresh 1:200 dilution of both fluorescein isothiocyanate-labeled goat anti-rabbit IgG (Cappel Laboratories) and Texas Red-labeled donkey anti-mouse IgG (Jackson ImmunoResearch) in PBS/3% BSA with 1% goat and donkey nonimmune serum. Coverslips were washed as above, then washed twice in PBS, and mounted onto a drop of diazobicyclo[2,2,2]octane solution (100 mg/ml in 90% glycerol/PBS with *p*-phenylenediamine at 1 mg/ml). Cells were observed with a Nikon Optiphot equipped with an MRC Lasersharp confocal imaging system (Bio-Rad). For microtubule-destabilizing conditions (28), cells were extracted at 4°C for 10 min in 0.1 M Pipes, pH 7.4/1% Triton X-100/80 μM $CaCl_2$ and fixed and stained as above.

RESULTS

Eukaryotes Have a 34-kDa Protein That Is Immunoreactive with Antibodies to 34-kDa CKI. Polyclonal rabbit antibodies were raised against the purified human erythrocyte 34-kDa CKI and affinity-purified on 34-kDa CKI covalently attached to Sepharose CL-4B. Western blotting indicated that the antibodies were highly specific, reacting only with a 34-kDa protein in lysates of all mammalian cells examined (Fig. 1 *Top*). This 34-kDa protein appears to be highly conserved, as an immunoreactive protein of the same size was detected in *Xenopus* oocytes, *Drosophila*, and *S. cerevisiae* (Fig. 1 *Middle*). In *S. cerevisiae*, a 34-kDa crossreactive protein copurified with membrane fractions (Fig. 1 *Middle*, lane F); mammalian CKI activity also copurified with membrane fractions (1–3). In all three organisms, crossreactive proteins of greater sizes were weakly detected. These larger proteins may represent related kinases, such as HRR25, YCK 1, and YCK 2 of *S. cerevisiae*, which are 50–60 kDa (23, 24).

To further demonstrate their specificity, the antibodies were used to immunoprecipitate the 34-kDa immunoreactive band, and the precipitate was assayed for CKI activity. The antibodies immunoprecipitated casein kinase activity from a solution of purified 34-kDa CKI and also from the erythrocyte cytosol (Fig. 1 *Bottom A* and *B*). Moreover, the antibodies removed the 34-kDa protein from cytosol, and the 34-kDa immunoreactive protein was detected in the immunoprecipitate by Western blotting and by casein kinase assay (Fig. 1 *Bottom B* and *C*).

Amino Acid Sequence of the Human Erythrocyte 34-kDa CKI. Internal amino acid sequence of the purified 34-kDa CKI was obtained from a lysine-specific proteolytic digest (Fig. 2). Sequence comparison (GenBank, November 1991) showed the human erythrocyte CKI to be closely homologous to six protein kinases and 98% identical to bovine α-CKI. This level of identity suggests that the 34-kDa CKI is the human form of α-CKI. The bovine α-CKI, in turn, is 62% identical to the catalytic region of HRR25 protein kinase from *S. cerevisiae* (23). However, α-CKI shows <25% identity with any other protein kinase, including human CDC2, casein kinase II, calcium/calmodulin-dependent kinase II, and cAMP-dependent protein kinase (22–24). Further, since all four of the peptides sequenced were from α-CKI, and no other sequence was detected, this suggests that the 34-kDa CKI preparation did not contain β-CKI, which is about the

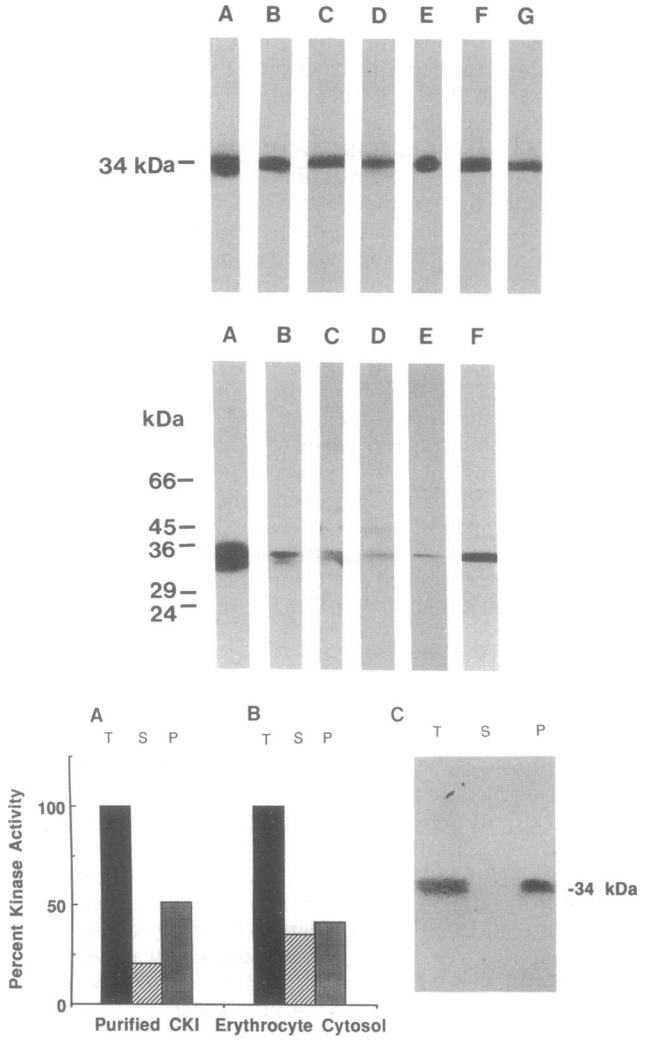

FIG. 1. (*Top*) Western immunoblot analysis with antibody to human erythrocyte 34-kDa CKI. Lane A, purified CKI (100 ng); lane B, erythrocyte lysate; lane C, CHO cell lysate; lane D, K562 cell lysate; lane E, A431 cell lysate; lane F, B82 cell lysate; lane G, BALB/c 3T3 cell lysate. For cell lysates, about 150 μg of protein was loaded. (*Middle*) Immunoblot analysis. Lane A, purified CKI (100 ng); lane B, K562 cells; lane C, *Xenopus* oocytes; lane D, *Drosophila*; lane E, total *S. cerevisiae* lysate; lane F, purified *S. cerevisiae* membranes. (*Bottom*) Immunoprecipitation of the 34-kDa CKI by affinity-purified anti-CKI antibodies. *A* and *B* show protein kinase activity toward casein: T, total; S and P, activities in the supernatant and pellet after immunoprecipitation of purified 34-kDa CKI (*A*) or of the 34-kDa CKI from erythrocyte cytosol (*B*). For *A*, 4-fold more 34-kDa CKI was used than in *B*. *C* shows a Western blot with CKI antibody corresponding to *B*. The majority of 34-kDa CKI in the cytosol was immunoprecipitated and was found in the pellet by Western blotting; this corresponds to the kinase activity in *B*. The remaining activity in the cytosol (S) was most likely due to casein kinase II.

same molecular mass. Since there was no sequence that was not α-CKI, this is additional evidence that the 34-kDa CKI preparation used for immunization and affinity purification of antibodies contained only the 34-kDa α-CKI. These results and the characterization of the affinity-purified antibodies are strong evidence that these antibodies are specific for the 34-kDa α-CKI.

Immunofluorescence Localization of α-CKI. Affinity-purified α-CKI antibodies were used for intracellular localization of α-CKI in B82 mouse fibroblasts and CHO cells. The labeling specificity was confirmed in experiments where immunofluorescent staining was blocked by preincubating antibody with purified α-CKI crosslinked to Sepharose or

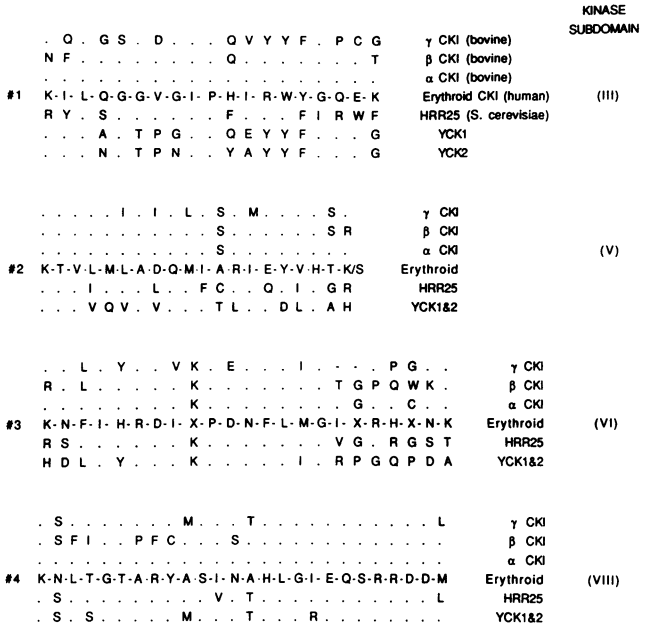

FIG. 2. Human erythroid CKI amino acid sequences and homologies with other similar protein kinases. Sequences were compared with the human erythrocyte 34-kDa CKI sequence by using GenBank; only nonidentical residues are shown. The locations of the sequences, when compared with the conserved protein kinase subdomains, are shown according to Hanks *et al.* (31).

with denatured CKI (Fig. 3 *a* and *b*). Cells treated with secondary antibodies alone showed no staining. In interphase cells, antibodies localized α-CKI to vesicular structures distributed throughout the cytosol (Fig. 3 *c* and *d*). Using a rat monoclonal antibody to BiP, an endoplasmic reticulum (ER)-localized protein, we found that a portion of the vesicular CKI staining colocalized with the ER (data not shown). This suggests that α-CKI associates with intracellular vesicular membranes and that portions of these vesicular structures contain BiP and thus are of ER origin.

The centrosome was also brightly stained in both interphase and mitotic cells, as shown in cells treated with nocodazole (Fig. 3 *e* and *f*). In interphase cells, no direct localization of CKI to microtubules was apparent, although the vesicular staining pattern in many cells clearly aligned with microtubules; this was particularly apparent when flat cells, such as LLC-PK pig kidney epithelial cells, were stained with α-CKI antibody (data not shown). The immunofluorescence localization of α-CKI is consistent with previous biochemical data showing that CKI activity was found associated with membranes and the cytoskeleton and also in the cytosol (1–3).

When cells entered mitosis, the vesicular staining was decreased compared with interphase cells, but the mitotic spindle and centrosome became intensely stained (Fig. 3 *g* and *h*). Extraction of CHO cells with microtubule-stabilizing buffer containing detergent, prior to fixation, preserved staining of the mitotic apparatus but somewhat reduced cytoplasmic staining (Fig. 3 *i* and *j*). Detergent extraction demonstrated that α-CKI was not artifactually associated with the mitotic apparatus as a result of fixing intact cells.

α-CKI Localizes to Kinetochore Fibers. As cells entered prophase, centrospheric staining intensified and staining of microtubules became apparent. Interestingly, in early prophase, CKI localized to all astral microtubules (data not shown), but later, in prophase/prometaphase, astral microtubules showed little detectable staining, whereas interpolar microtubules remained stained (Fig. 4 *a* and *b*). In metaphase, the staining of spindle microtubules was very intense (Fig. 4 *c* and *d*). To determine whether staining of spindles was on the kinetochore microtubules or other interpolar

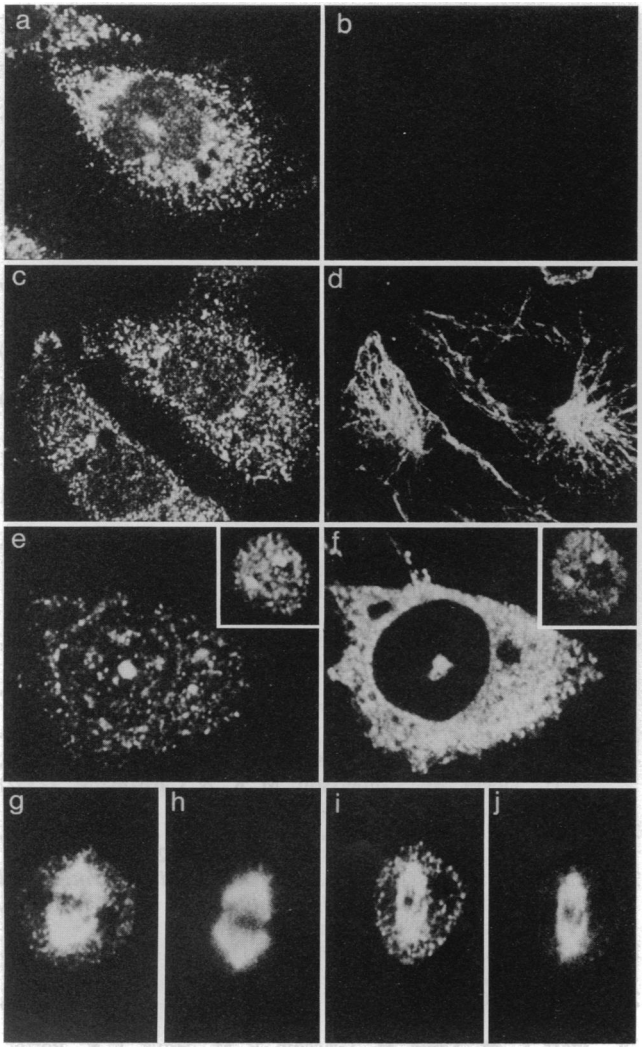

FIG. 3. Localization of α-CKI in intact interphase and dividing CHO cells. Row 1: interphase cell stained with anti-CKI antibody (*a*) and interphase cell stained with anti-CKI antibody that was preincubated with CKI-Sepharose (*b*); antibody preincubated with electroeluted CKI showed only background staining. Row 2: interphase cells. Row 3: interphase and mitotic (*Inset*) cells treated with nocodazole. Row 4: *g* and *h*, intact mitotic CHO cell; *i* and *j*, mitotic CHO cell extracted with 0.5% Triton X-100. Cells were stained for CKI (*a*, *b*, *c*, *e*, *g*, and *i*) or β-tubulin (*d*, *f*, *h*, and *j*). (*a–f*, ×700; *g–j* and *Insets*, ×1400.)

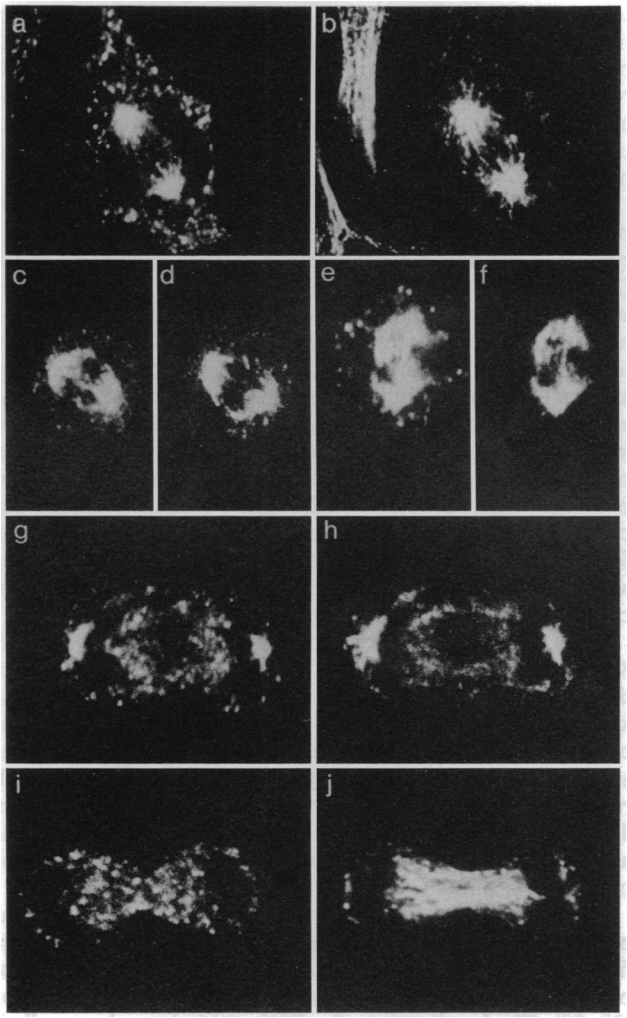

FIG. 4. Localization of α-CKI in cells progressing through mitosis. Row 1: prometaphase B82 cell. Row 2: *c* and *d*, metaphase B82 cell; *e* and *f*, metaphase CHO cell extracted, before fixation, under conditions designed to destabilize all but kinetochore microtubules. Row 3: anaphase cell. Row 4: telophase cell. Cells were stained for CKI (*a*, *c*, *e*, *g*, and *i*) and β-tubulin (*b*, *d*, *f*, *h*, and *j*). All cells (except *e* and *f*) were intact B82 cells when fixed. (×700.)

microtubules, CHO cells were extracted with 1% (vol/vol) Triton X-100 in buffer at 4°C in the presence of 80 μM $CaCl_2$. This procedure depolymerizes all but kinetochore microtubules (28). CHO cells treated in this manner still showed spindle staining with α-CKI antibodies, suggesting that α-CKI was localized to kinetochore microtubules during mitosis (Fig. 4 *e* and *f*). However, this does not exclude the possibility that α-CKI localized on polar microtubules as well. The localization to microtubules diminished in anaphase cells (Fig. 4 *g* and *h*), and there was no detectable midbody microtubule localization. In telophase cells, midbody microtubules did not stain with CKI antibody; the only microtubule localization was around the centrosome. Vesicular staining was also recovered in telophase (Fig. 4 *i* and *j*). When A431 human epidermal carcinoma cells and LLC-PK cells were stained with CKI antibodies, identical results were obtained (data not shown).

DISCUSSION

Previously, the membrane association and protein kinase activity of the erythroid 34-kDa CKI have been shown to be regulated by PIP_2 (2, 3). Here we show that, in interphase cells, α-CKI is localized to vesicular cytosolic structures. These vesicular structures colocalize with structures that contain ER markers, and the vesicular structures containing α-CKI are often aligned with microtubules.

Recently, it has been shown that PIP_2 is synthesized on the ER and other intracellular membranes (32). Thus, vesicular localization of α-CKI may be regulated by PIP_2, as previously shown for CKI *in situ* (2, 3). Regulation of proteins that associate with membranes may prove to be a general regulatory mechanism, since a number of proteins and enzymes, including tyrosine phosphatases, actin-binding proteins, protein 4.1, and protein kinase C, may also be regulated by PIP_2 (13–21).

When cells enter prophase, the localization of α-CKI increases around the centrosome, and there is uniform colocalization of α-CKI with the microtubule aster. As cells progress into mitosis, the α-CKI becomes localized to mitotic spindle fibers and there is no longer detectable astral microtubule localization. Moreover, in mitosis, α-CKI localizes to kinetochore fibers. As cells progress into anaphase, α-CKI localization appears restricted to regions of microtubules between the centrosome and the chromosomes. The α-CKI localization on microtubules decreases as cells progress to

telophase. In telophase, α-CKI is localized to the centrosome, but there is no localization to midbody microtubules. Taken together, these results suggest that α-CKI relocalizes from vesicular structures to the centrosome and aster microtubules in prophase and with kinetochore microtubules and possibly other interpolar microtubules during mitosis.

The HRR25 protein kinase from yeast is homologous to the human α-CKI (22, 23). The yeast protein kinase appears to regulate chromosomal segregation, meiosis, and DNA repair (23). Deletion of the HRR25 gene results in a phenotype in which yeast cells undergo division but are defective in the ability to segregate chromosomes (23). The intracellular localization of the human α-CKI to the centrosome and kinetochore microtubules is consistent with a role for human α-CKI in chromosomal separation similar to HRR25 function in yeast. The intracellular relocation of CKI upon the onset of mitosis may be induced by phosphorylation catalyzed by enzymes, such as the CDC2 protein kinase, that are cell cycle-regulated (33, 34). Other alternatives are also possible; for example, different forms of α-CKI may be generated by alternative mRNA splicing (22), and one of these isoforms could associate with the mitotic spindle.

The CDC2 protein kinase localizes to mitotic spindles (35, 36), as does casein kinase II (37, 38) and calcium/calmodulin-dependent protein kinase II (39); cAMP-dependent protein kinase type II is associated with centrosomes (40). The combined activity of these protein kinases may be required to sequentially, or hierarchically, phosphorylate proteins (41) required for spindle assembly and chromosomal separation. The interaction of protein kinases with the centrosome may also play a role in centrosomal regulation of the progression of cells into mitosis (42).

Possible cellular targets for phosphorylation by CKI may be kinesin or dynein, which are localized to kinetochore fibers and the kinetochore during mitosis and are thought to provide the mechanical force for chromosomal separation (29, 30). The localization of α-CKI within interphase cells is also similar to that of kinesin and dynein (43–45). In conclusion, our results indicate that the CKI family of protein kinases appears to be structurally and functionally conserved from humans to yeast. In eukaryotes, it appears likely that α-CKI regulates, and is regulated by, the cell cycle.

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