Cell cycle independent interaction of CDC2 with the centrosome, which is associated with the nuclear matrix-intermediate filament scaffold

(cyclin-dependent kinases/growth control/osteoblasts/immunoelectron microscopy/pericentrin)

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ABSTRACT The cell cycle regulating Cdc2 protein kinase helps orchestrate cell cycle dependent changes in cell structure and function. This report shows that Cdc2 is localized to the centrosome region and is tightly bound to the nuclear matrix-intermediate filament scaffold. Antibodies to Cdc2 and to the centrosome-specific protein, pericentrin, label the centrosome in an apparently cell cycle independent manner. Isolated centrosomes also label similarly with both antibodies. Essentially, all cells show Cdc2 labeling of the centrosomes, implying an independence of the stage in the cell cycle, a conclusion supported by studies of synchronized cells. In contrast to the labeling of every cell with the Cdc2 monoclonal antibody, fewer centrosomes were labeled with an antibody to the PSTAIRE domain of Cdc2. Embedment-free, immunogold electron micrographs of extracted cell whole mounts show the centrioles and a pericentriolar network of filaments. Both Cdc2 and pericentrin antibodies decorate the amorphous pericentriolar material, while the Cdc2 antibodies also decorate the centrioles themselves. The constitutive presence of Cdc2 at the centrosome suggests a continuing role in the dynamics of centrosome function throughout the cell cycle.

Cyclins and cyclin-dependent kinases (cdks) control cell cycle progression by phosphorylating regulatory proteins (1–7). In addition, these cyclin-related proteins appear to affect cell structure and function independent of the cell cycle. Cyclins and cdkS have a role in the development and maintenance of cell- and tissue-restricted properties of differentiated cells, including osteoblasts (8), myoblasts (9), neurons (10), and hematopoietic cells (11).

Regulatory activities of cdkS and cyclins occur in several subcellular compartments (12). For example, the localization of human cyclin B1 in the nucleus or cytoplasm has been shown to be cell cycle dependent (13), whereas human cyclin B2 is primarily associated with the Golgi apparatus (14). Here we demonstrate that Cdc2/Cdk1 interacts with the centrosome in a cell cycle independent manner and is associated with the nuclear matrix-intermediate filament cellular scaffold.

MATERIALS AND METHODS

Cell Culture. WI-38 human diploid lung fibroblasts were grown in DMEM medium containing 10% fetal calf serum (FCS). HeLa human cervical epithelial carcinoma cells were plated in DMEM medium containing 5% FCS and 5% horse serum. IMR-90 human diploid lung fibroblasts were plated in BME medium with 10% FCS. Saos-2 human primary osteogenic sarcoma cells were plated in Mccoys 5A medium containing 15% FCS. All culture media were supplemented with 200 mM L-glutamine and 500 mM penicillin/streptomycin. Cells were plated on 0.5% gelatin-coated glass cover slips. All cells were harvested during exponential phase growth.

Cell Synchrony. Two procedures were employed for cell synchrony. For biochemical synchronization, a double thymidine block was carried out (15). Cells were harvested at time points representing key cell cycle phases: 0 (G1/S), 2 (early S), 4 (mid S), 8 (G2/M), 11–12 (G1) hours after release from the second block. The cells were extracted with detergent so as to image the cytoskeleton and further processed to reveal the nuclear matrix intermediate filament scaffold (NM-IF).

For selection synchronization, cells were incubated with the Hoechst 33342 fluorescent DNA dye [37°C for 1 hr in 10 mM Na2HPO4/1 mM KH2PO4/137 mM NaCl/2.7 mM KCl (PBS)/5% FCS]. Cells were sorted by DNA content into three cell cycle stages: G0/G1, S, and G2/M using fluorescence-activated cell sorting with a MoFlo flow cytometer (Cytometry, Fort Collins, CO). Cells were immediately collected by a Shandon Cytospin 3 onto gelatin-coated coverslips and processed to examine the cytoskeleton and the NM-IF. The preparations were then stained for immunofluorescence analysis.

Cytoskeletal and NM-IF Preparations (16). All cover slips were rinsed twice on ice with PBS, then extracted twice with cytoskeletal (CSK) buffer containing 2 mM vanadyl ribonucleoside complex (VRC) for 3 min each, then once for 3 min with CSK buffer without VRC. Cytoskeletal preparations were fixed in 3.7% formaldehyde in CSK buffer for 10 min while gradually warming to room temperature, followed by two rinses in PBS, two rinses in PBS with 0.5% bovine serum albumin (PBS-A), and then stored at 4°C until antibody staining.

After the rinse in CSK buffer, the NM-IF was prepared by digestion with DNase I (100 mg/ml) in digestion buffer (CSK buffer with 50 mM NaCl) twice for 10 min at room temperature. The final extraction was performed in digestion buffer containing 0.25 M (NH4)2SO4 twice for 5 min at 0°C. Cells were fixed in 3.7% formaldehyde in digestion buffer for 10 min at room temperature, rinsed twice in PBS and twice in PBS-A.

Centrosome Isolation Procedure. HeLa cells were incubated for 90 min at 37°C in 10 mg/ml nocodazole and cytochalasin B, which depolymerize microtubule and actin filaments, respectively. The cells were washed in PBS, PBS and 8% sucrose, and 8% sucrose and then lysed in 1 mM Tris (pH 8)
8), 0.1 mM 2-mercaptoethanol, 0.5% Nonidet P-40. The lysates were filtered through Nytex (40 μm), transferred to Corex tubes, underlaid with 20% Ficoll and centrifuged at 25,000 × g for 15 min at 2°C. The clear interface was collected and brought to a final concentration of 10 mM Pipes, 1 mM EDTA, 8 mM 2-mercaptoethanol (pH 7.2). The centrosome preparations were then processed for immunofluorescence as described (17) using 3.7% formaldehyde in CSK buffer (16).

Immunofluorescence Antibody Staining. Fixed coverslips were incubated for 1 hr at 37°C with primary antibodies. Antibodies used were Cdc2 p34 (17) (1:100 dilution, mouse monoclonal IgG2; Santa Cruz Biotechnology no. SC-54), Cdc2 p34 (H-297) (1:50 dilution, affinity-purified rabbit polyclonal IgG; Santa Cruz Biotechnology no. SC-747), Cdc2 PSTAIRE (1:500 dilution, rabbit polyclonal IgG; Santa Cruz Biotechnology no. SC-53), pericentrin 4B (1:200 rabbit polyclonal), and 5051 (1:500 dilution, human scleroderma autoimmune serum to centrosome) (17). Secondary antibodies including Texas Red-conjugated donkey anti-mouse antibody (1:500, Jackson ImmunoResearch), Texas Red-conjugated donkey anti-rabbit antibody (1:100, Jackson ImmunoResearch), fluorescein isothiocyanate-conjugated goat anti-rabbit antibody (1:100, Jackson ImmunoResearch), or a rhodamine-conjugated affinity-purified goat anti-human (1:100, Cappel) were incubated for 1 hr at 37°C. DNA content was evaluated using 4′,6-diamidino-2-phenylindole (DAPI) (5 μg/ml, Sigma) dissolved in PBS containing BSA and 0.1% Triton X-100 to evaluate the efficacy of DNA removal during the NM-IF extraction. After washing with PBS, the coverslips were mounted on 3 × 1 slides using Vectashield H-1000 (Vector Laboratories). Cells were observed using a Zeiss ICM 405 microscope with epifluorescence, 100× Zeiss objective. Color slides were taken with Kodak Ektachrome 400 film. For high resolution analyses, digitized images were collected with a Photometrics series 200 charge-coupled device camera using a high resolution, shallow depth-of-field 100× objective (numerical aperture = 1.4, Zeiss) and customized computer software.

Immunogold Labeling of Embedment-Free Cell Whole Mounts. Saos cells were grown on parlodian films on carbon-coated sterile nickel grids (Ted Pella, Redding, CA). The soluble proteins were removed by extracting the cells for 3 min at 4°C in CSK buffer containing 0.5% Triton, VRC, and protease inhibitors (16). The extracted cells were fixed for 30 min in 4% paraformaldehyde/0.5% glutaraldehyde [Ted Pella, electron microscopy (EM) grade], at 4°C, and subsequently rinsed twice in the cytoskeletal buffer. The cells on grids were washed twice in TBS-I (18) and incubated for 10–20 min in 10% normal goat serum (NGS, Sigma) at room temperature. They were then incubated either overnight at 4°C, or for 1–2 hr at 37°C in a mixture of antibodies containing monoclonal anti-Cdc2 and polyclonal anti-pericentrin 4B diluted in 2% NGS in TBS-I (18). The stained cells were then washed several times with TBS-I and incubated for 10–40 min. The grids were incubated for 1 hr in a mixture of secondary antibodies coupled with colloidal gold, diluted in TBS-II (18) (goat anti-mouse IgG/15 nm gold and goat anti-rabbit IgG/5 nm gold Auroprobe-EM, Amersham). The cells were then rinsed three times in TBS-I. Following immunostaining, the samples were fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate (pH 7.4) for 30 min, washed in 0.1 M cacodylate buffer, dehydrated in ethanol, and critical point dried. The samples were thinly coated with carbon and viewed using a JEOL 1200 EX electron microscope. A total of 450 centrosomes were examined in 5 separate preparations.

RESULTS

Cdc2 Is Associated with the Centrosome and Retained in the NM-IF. Fig. 1 shows the immunofluorescence analysis of detergent-extracted cells grown on coverslips. Immunofluorescence using anti-Cdc2 antibody showed prominent fluorescent signals in the centrosome region as well as a much weaker, broadly distributed cytoplasmic labeling and a nuclear labeling whose intensity depends on cell type. Identification of centrosomes was directly established by using antibodies against the centrosome-specific protein pericentrin. The labeling of Cdc2

![Fig. 1. Association of Cdc2 with the centrosome in Saos-2 human osteosarcoma cells. (Left) Immunostaining was carried out using Cdc2 mouse monoclonal antibody (red) and a pericentrin rabbit polyclonal antibody (green) on detergent extracted cells (Upper) or NM-IF preparations (Lower). The colocalized signals are yellow. (Right) DAPI staining shows presence of DNA in nuclei of detergent-extracted cells (Upper) but not in NM-IF preparations (Lower).](image1)

![Fig. 2. Immunostaining of centrosomes with a Cdc2 mouse monoclonal antibody in detergent-extracted exponentially growing WI-38 human diploid fibroblasts. The centrosomes are designated by arrowheads.](image2)
and pericentrin is almost completely coincident when viewed using a dual-band pass filter (Fig. 1).

Both pericentrin and Cdc2 staining remained localized at the centrosomes in an NM-IF preparation. The detergent-extracted cells were digested with DNase I (Fig. 1) and extracted with 0.25 M (NH₄)₂SO₄, which removes chromatin and many of the cytoskeletal filaments (16). The absence of detectable DAPI staining indicates that most DNA was removed. Retention of Cdc2 signal after extraction with high ionic strength ammonium sulfate indicates that Cdc2 is tightly associated with structural elements of the centrosome. This association occurs in a variety of exponentially growing cells with a broad spectrum of phenotypic properties. Also, essentially every cell in cultures of tumor-derived (Saos-2 and HeLa) and normal (WI-38 and IMR-90) human cell lines showed Cdc2 bound to the centrosome (Fig. 2 and data not shown).

The more widely distributed labeling of Cdc2 antigen in the cytoplasm and nucleus of cells appears genuine and not a background artifact. Interestingly, Cdc2 labeling is somewhat stronger in the nuclei of normal diploid cells when compared with similarly extracted tumor cells.

**Immunolocalization Specificity.** We performed a series of control experiments to establish specificity of Cdc2 labeling. Staining required the presence of both primary and secondary antibodies. The labeling pattern was unaffected when the fluorochromes conjugated to the secondary antibodies were reversed. Closely similar labeling of centrosomes was obtained with very different antibodies. For example, both detergent-

![Fig. 3. Immunostaining of centrosome preparations from exponentially growing HeLa cells. Immunolabeling was carried out with a Cdc2 mouse monoclonal antibody (red, Left), pericentrin rabbit polyclonal antibody (green, Center), or both Cdc2 and pericentrin (Right).](image)

![Fig. 4. EMs of immunostained Saos whole-mount CSKs. (B) An enlarged area of A. The arrowheads point to the two centrioles. The large gold beads (15 nm) show the distribution of Cdc2 and the small beads (5-nm gold beads) the pericentrin antigen. ([A] bar = 500 nm, [B] bar = 100 nm.)](image)
extracted cytoskeleton and salt extracted NM-IF preparations showed localization of Cdc2 at the centrosome region when using an affinity-purified rabbit polyclonal Cdc2 antibody and a human autoimmune antiserum recognizing centrosomes.

**Cdc2 Association with Isolated Centrosomes.** So far, Cdc2 has been found to colocalize with the centrosome-specific antigen, pericentrin. The two antigens also copurify in centrosome-enriched preparations, suggesting that they are bound to identical or linked structures. Fig. 3 shows that the centrosomes, isolated by centrifugation onto a 20% Ficoll cushion, label with antibodies to both Cdc2 and pericentrin (Fig. 3). A small subset of isolated centrosomes, which shows only a single antigen may reflect dissociation of Cdc2 during the isolation procedure.

**Ultrastructural Immunogold Localization of Cdc2.** Immunofluorescence shows the approximate colocalization of Cdc2 and pericentrin proteins to within the resolution of the optical microscope. This suggests, but does not incontrovertibly prove, that Cdc2 and pericentrin are both bound to elements of the centrosomes. The much greater resolving power of the electron microscope would allow a more precise immunolocalization, but conventional EM shows the centrosome region only as a vague “pericentriolar cloud.” However, embedding-free EM shows the centrioles and the surrounding structures with great clarity and allows gold bead conjugated antibodies to locate specific antigens with improved precision.

The extracted cell whole mount in the micrograph in Fig. 4 shows the immunogold labeling of the centrosome region with two antibodies, one against Cdc2 and the other against pericentrin. Two complete centrioles, marked by arrowheads, lie

![Fig. 5](image_url)
centromeres only during early G1 phase (Fig. 5D), but not during other cell cycle phases (Fig. 5C and data not shown). High resolution digital imaging analysis of early G1 cells clearly indicates juxtaposition, but not complete overlap, of Cdc2 and PSTAIRE signals at the centrosome (Fig. 6). Thus, the detection of Cdc2 at the centrosome is antibody dependent (see Discussion).

**DISCUSSION**

We have shown, using two Cdc2-specific antibodies, that the Cdc2/Cdk1 protein is associated with centrosomes throughout the cell cycle in several different cell types. We obtained positive in situ immunofluorescence signals with both monoclonal and polyclonal Cdc2-specific antibodies using exponentially growing cells, as well as cells synchronized by sorting or induced synchrony. Furthermore, our observation that Cdc2 and pericentrin are associated in almost all isolated centrosomes validates the in situ association of Cdc2 with the centrosome in intact cells. Thus, multiple lines of evidence support cell cycle independent association of Cdc2 with the centrosome.

In contrast to the results obtained with the Cdc2 antibody, we observed that centrosome labeling with antibodies directed against the conserved PSTAIRE domain of Cdc2 is strongly cell cycle dependent, as previously reported by Bailly et al. (19). This may result from the PSTAIRE epitope of Cdc2 being inaccessible during specific stages of the cell cycle, which would explain the difference between the results of Bailly et al. and those obtained here.

Our results show that the centrosomal structures are bound to the NM-IF scaffold. The resinless section electron micrographs show both the centrioles and the surrounding material enmeshed in filaments of the NM-IF. Although centrosomes are known to associate with and organize microtubules, centrosome binding to the NM-IF is not through the microtubules, since these are absent from the NM-IF preparation. The electron micrographs also indicate that Cdc2 is localized somewhat differently than pericentrin, consistent with spatial specialization within the centrosome. Functional involvement of Cdc2 with reorganization and spatial redistribution of centrosomes during cell cycle progression remains to be established. However, association of Cdc2 with the centrosome may have a role in the dynamic interrelationships between the centrosome and cytoarchitecture.

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