

Association of 14-3-3 Proteins with Centrosomes

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ABSTRACT: The 14-3-3 proteins are involved in diverse signal transduction pathways and interact physically with a wide variety of proteins. Here, we report the partial sequence analysis of a human spleen 14-3-3 protein, which was identified as a variant form of the ϵ isoform. A peptide antibody generated to the variant 14-3-3 localizes in the centrosome and spindle apparatus of mouse leukemic FDCP cells by immunofluorescence microscopy. Immunoblots of centrosomes isolated by sucrose density gradient centrifugation of cell lysates disclose only the ϵ and γ isoforms, while total cellular lysates contain the ϵ , γ , β and ζ isoforms of 14-3-3. These data suggest that a subset of total cellular 14-3-3 proteins are localized in the centrosomes and spindle apparatus. A differential localization of the centrosomal 14-3-3 was observed in mouse 3T3 cells. Serum-starved (quiescent) cells lack the centrosomal 14-3-3, but upon serum-stimulation of these quiescent cells, the centrosomal 14-3-3 reappears. We propose that a subset of intracellular 14-3-3 proteins are localized in the centrosome and spindle apparatus, and may in fact, link mitogenic signaling, the cell cycle, and perhaps the centrosome duplication cycle as well.

Keywords: 14-3-3 proteins, centrosomes, signal transduction, cell cycle

INTRODUCTION

The 14-3-3 family of proteins comprises a group of highly conserved, acidic proteins that have diverse intracellular functions. This family contains about 11 different isoforms that have been identified in mammals, amphibians, insects, plants, and yeast (1). Seven distinct isoforms have been identified in mammals (β , γ , ϵ , ζ , η , τ , Θ). In addition, the α and δ isoforms are the phosphorylated forms of β and ζ , respectively (2,3).

14-3-3 proteins were first described as activators of tryptophan hydroxylase involved in neurotransmitter biosynthesis, as well as modulators of protein kinase C (1). Recent studies have also suggested a role for 14-3-3 proteins in intracellular signaling and cell proliferation: (i) 14-3-3 proteins associate with the Raf-1 protein kinase, a component of the mitogen-activated protein (MAP) kinase cascade (4). (ii) several 14-3-3 isoforms are

differentially expressed in normal and transformed cells (5); (iii) 14-3-3 proteins associate with the polyoma virus middle T antigen (MT) in transformed cells (6); (iv) two yeast genes named *rad24* and *rad25* encode 14-3-3 protein homologs that are required for the DNA damage checkpoint before cells enter mitosis (7). Additional evidence in support of a role for 14-3-3 in the cell cycle and mitogenic signaling is the interaction of 14-3-3 ϵ and 14-3-3 β with the *cdc25A* and *cdc25B* phosphatases, whose activity is required for cell cycle progression (8).

Relatively little is known about the intracellular localization of 14-3-3 proteins. The β and ζ isoforms of 14-3-3 interact with protein kinase Raf, which upon activation is translocated to the plasma membrane (9). Several isoforms have also been detected in the Golgi apparatus (10). In adrenal chromaffin cells, 14-3-3 proteins stimulate the exocytosis of chromaffin granules and are

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associated with phospholipids and the actin cytoskeleton (11).

In this paper, we report the partial sequence analysis of human spleen 14-3-3 proteins and determined that a subset of cellular 14-3-3 proteins are associated with centrosomes. In addition, we show in 3T3 cells that the localization of 14-3-3 proteins in the centrosome is dependent on serum stimulation. The possible functional consequences of these findings for the role of 14-3-3 proteins in centrosome function and the cell cycle are discussed.

MATERIALS AND METHODS

Sequence Analysis of 14-3-3 Proteins

14-3-3 proteins were purified from human spleen during the course of purification of a phospholipid-stimulated protein kinase (12). Aliquots of a Mono Q preparation were subjected to direct cleavage with CNBr or digestion with trypsin of protein electroblotted onto nitrocellulose membrane. Peptides were purified by HPLC (13). Six CNBr peptides and three tryptic peptides were sequenced using an Applied Biosystems 470A gas-phase sequencer at the Protein Chemistry facility at the University of New Mexico School of Medicine and the Microchemistry Core Facility at Memorial Sloan-Kettering Cancer Center, New York, NY. Sequences were analyzed using the Genetics Computer Group (Madison, WI) software package.

Anti-peptide Antibody production and Immunoblotting

The peptide MDVELDVEERNLLSVAY (Sp4) was coupled via its C-terminal tyrosine (Y) to keyhole limpet hemocyanin (Pierce, Rockford, IL) using benzidine (14). Immunization of rabbits and serum collection were performed by BAbCO (Richmond, CA). Sera were tested for Sp4 specific antibody by ELISA using immobilized Sp4 pep-

tide. Sp4 peptide-specific antibodies were purified from sera by affinity chromatography (14) using covalently linked Sp4 peptide (AminoLink agarose, Pierce, Rockford, IL). Immunoblots were prepared by separation of cell lysates, subcellular fractions and 14-3-3 proteins by SDS-PAGE and transferred to PVDF membranes (15). The blots were incubated overnight at 4°C in antibody buffer (5% non-fat dry milk, 0.01% Tween-20, 1 mM CaCl₂ in TBS (16), followed by antibodies for 1 hr at 20°C. Bound antibodies were disclosed by incubation for 30 min with a goat anti-rabbit IgG or anti-mouse IgG horseradish peroxidase conjugates diluted 1:5000 in antibody buffer and ECL detection (Amersham, Arlington Heights, IL).

Cell Culture

Mouse FDCP1 leukemic cells were grown in RPMI medium containing 10% fetal bovine serum (Gibco-BRL, Gaithersburg, MD) and supplemented with 10% WEHI-conditioned medium as a source of interleukin 3. Mouse 3T3 cells were maintained in Dulbecco's modified Eagle's medium containing 10% bovine calf serum.

Immunofluorescence

Approximately 1×10^4 cells were attached to poly-L-lysine coated glass slides and fixed in acetone. Coverslips with adherent 3T3 cells were rinsed in PBS and fixed in acetone. FDCP cells and 3T3 cells on coverslips were treated with 3% BSA in PBS and then incubated with antibodies to Sp4, centrin, tubulin, and α -mannosidase II diluted in 1% BSA in PBS. In the competition experiment, affinity-purified Sp4 antibody was preincubated with a 50-fold molar excess of Sp4 peptide for 2 hours before adding to the cells. Bound antibodies were visualized with fluorescein conjugated goat anti-rabbit IgG (Fab'₂) (Zymed, South San Francisco, CA) and Texas-Red conjugated goat anti-mouse IgG (Fab'₂) (Cappel,

West Chester, PA). Slides were mounted on coverslips with 90 % glycerol in PBS, pH 9.0 containing 2.5 mg/ml 1,4-diazabicyclo-[2.2.2] octane (DABCO) and viewed in a Leitz microscope equipped for epifluorescence. The cells were photographed using Kodak T Max 400 film. Mouse 3T3 cells were plated at an initial density of 2×10^4 cell/ml on 22 mm square glass coverslips in 35 x 10 mm dishes with Dulbecco's modified Eagle's medium (DME) supplemented with 10% bovine calf serum. Quiescent cells were obtained by plating on glass coverslips in DME medium with 10 % serum and after 24 hr the medium was changed to DME with 0.5 % serum. After 48 hr in low serum medium, the cells were re-exposed to DME medium with 10 % serum for the indicated times.

Subcellular Fractionation

FDCP cells were lysed by boiling in 2.5 % SDS, 0.5 % NP-40, 0.5 % sodium deoxycholate in 50 mM Tris-HCl, pH 7.5 and then diluted with 9 volumes of 50 mM Tris-HCl, pH 7.5, 0.2 M NaCl, 6 mM EDTA, 2.5 % Triton X-100, and 100 units/ml of aprotinin (TNET). An aliquot was used for immunoprecipitation with 15 μ g/ml rabbit anti-centrin polyclonal antibody or 15 μ g/ml non-immune rabbit IgG for 15 hours at 4°C, followed by Protein A-Sepharose for another 60 min at 4°C. The beads were washed five times with TNET and were incubated in Laemmli sample buffer at 56°C for 20 min. The eluted proteins were analyzed by immunoblotting with mouse monoclonal anti-centrin and horseradish peroxidase conjugated sheep anti-mouse IgG followed by ECL detection.

Centrosomes were prepared from 2×10^9 FDCP-1 cells as described for human lymphoblastic KE37 cells (17). The yield of total centrosomal protein (2 μ g) from 2×10^9 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 (18).

RESULTS

Sequence Analysis of Human Spleen 14-3-3 Proteins and Characterization of 14-3-3 Anti-peptide Antibody

We purified and partially sequenced human spleen 14-3-3 proteins, which co-purified with a phospholipid-stimulated protein kinase (12). Two peptides, Sp1 and Sp4 (Fig. 1) were found to be homologous to known 14-3-3 isoforms (1). The Sp1 peptide is identical to the ζ 14-3-3 isoform. The Sp4 peptide contains a highly conserved region (EERNLLSVAYKNVIGA) (1). However, the first 7 residues at the amino terminus (MDVELDV) of Sp4 are more closely related to the ϵ isoform from sheep and mouse brain, as well as yeast 14-3-3, but contains a single amino acid change (T→D) from these 14-3-3 proteins. To further characterize and study the intracellular distribution of the Sp4 14-3-3 protein, an antiserum was prepared to the peptide, DVELDVEERNLLSVAY in Sp4 (Fig. 1). The specificity of the affinity-purified Sp4 (14-3-3)-IgG was ascertained by immunoblotting of partially purified 14-3-3 proteins isolated from human acute myelogenous leukemic (AML) cells and bovine adrenal gland; a 30/32 kDa doublet is recognized by the antibody in the human AML 14-3-3 preparation (Fig. 1A). A polyclonal anti-14-3-3 serum raised against bovine adrenal 14-3-3 proteins (19) discloses multiple bands in bovine adrenal gland 14-3-3 (Fig. 1 B, C), corresponding to the different 14-3-3 isoforms, while the anti-Sp4 (14-3-3) antibody recognizes only a single band (Fig. 1D).

Immunolocalization of 14-3-3 Proteins in FDCP Cells

We next studied the intracellular location of the 14-3-3 isoform recognized by the affinity-purified Sp4 antibody by immunofluorescence microscopy of cells. Highly localized staining was observed in the perinuclear region (Fig. 2A), suggesting that either the centrosome or the Golgi

	31		61
Sp1		MKSVTEQGAELSNEERNLLSVAYKNVGA	
Sp4	MDVELDVEERNLLSVAYxNVIGA	
ε		MKKVAGMDVELTVEERNLLSVAYKNVIGA	
ζ		MKSVTEQGAELSNEERNLLSVAYKNVGA	
β		MKAVTEQGHLSNEERNLLSVAYKNVGA	
γ		MKNVTELNEPLSNEERNLLSVAYKNVGA	
η		MKAVTELNEPLSNESRNLLSVAYKNVGA	
τ		MKAVTEQGAELSNEERNLLSVAYKNVGG	
D		MKSVTETGVELSNEERNLLSVATKNVGA	
Y		MKTVASSGQELSVEERNLLSVAYKNVIGA	

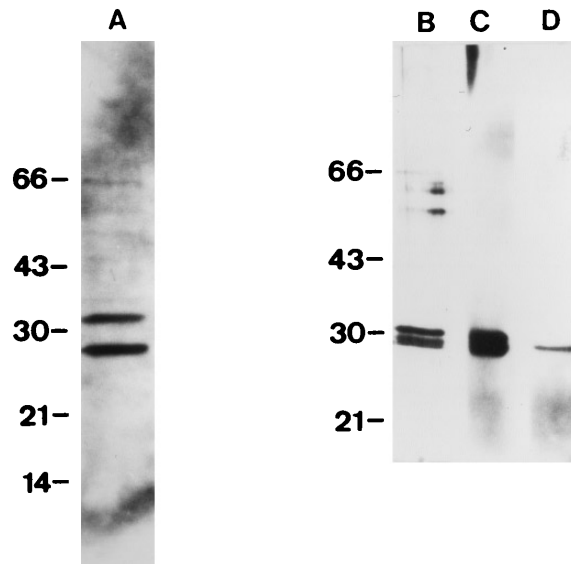


Figure 1. A peptide-specific antibody to human spleen 14-3-3 recognizes a subset of 14-3-3 proteins. The Sp1 and Sp4 peptides from the human spleen 14-3-3 preparation are shown in alignment with 14-3-3 isoforms in a region encompassing residues 31 through 61 (1). 14-3-3 proteins from human AML cells (A) were immunoblotted with affinity-purified anti-Sp4 (14-3-3) IgG. 14-3-3 proteins from bovine adrenal gland (B,C,D) were separated by SDS-PAGE and visualized by silver staining (B), immunoblotting with polyclonal anti-bovine adrenal gland 14-3-3 serum (C), and affinity-purified anti-Sp4 (14-3-3) IgG (D).

apparatus contained the antigen recognized by anti-Sp4 (14-3-3) IgG, while the preimmune serum gave no such staining pattern (now shown). As shown in Figure 2A and B, the staining observed with anti-Sp4 (14-3-3) IgG colocalizes with antibody to the pericentrosomal protein, centrin (20). In double label immunofluorescence using anti-centrin and an antibody to a Golgi-specific enzyme, α -mannosidase II, the Golgi cisternae are oriented around the centrosomal region (Fig. 2C and D). This orientation is consistent with the electron microscopic localization of

the centrosome and the Golgi complex in early myeloid progenitor cells (21). Additional evidence in support of a centrosomal localization of the Sp4 14-3-3 and not the Golgi apparatus is seen in Figure 2A, where anti-Sp4 (14-3-3) labels both centrosomes and the spindle fibers of the metaphase cell (arrows). The labeling at the spindle poles co-localizes with anti-centrin, whose labeling is restricted to centrosomes (Fig. 2B, arrows), but not spindle fibers (20). This pattern of labeling by anti-Sp4 (14-3-3) and anti-centrin in metaphase cells is consistent with a centrosomal localization

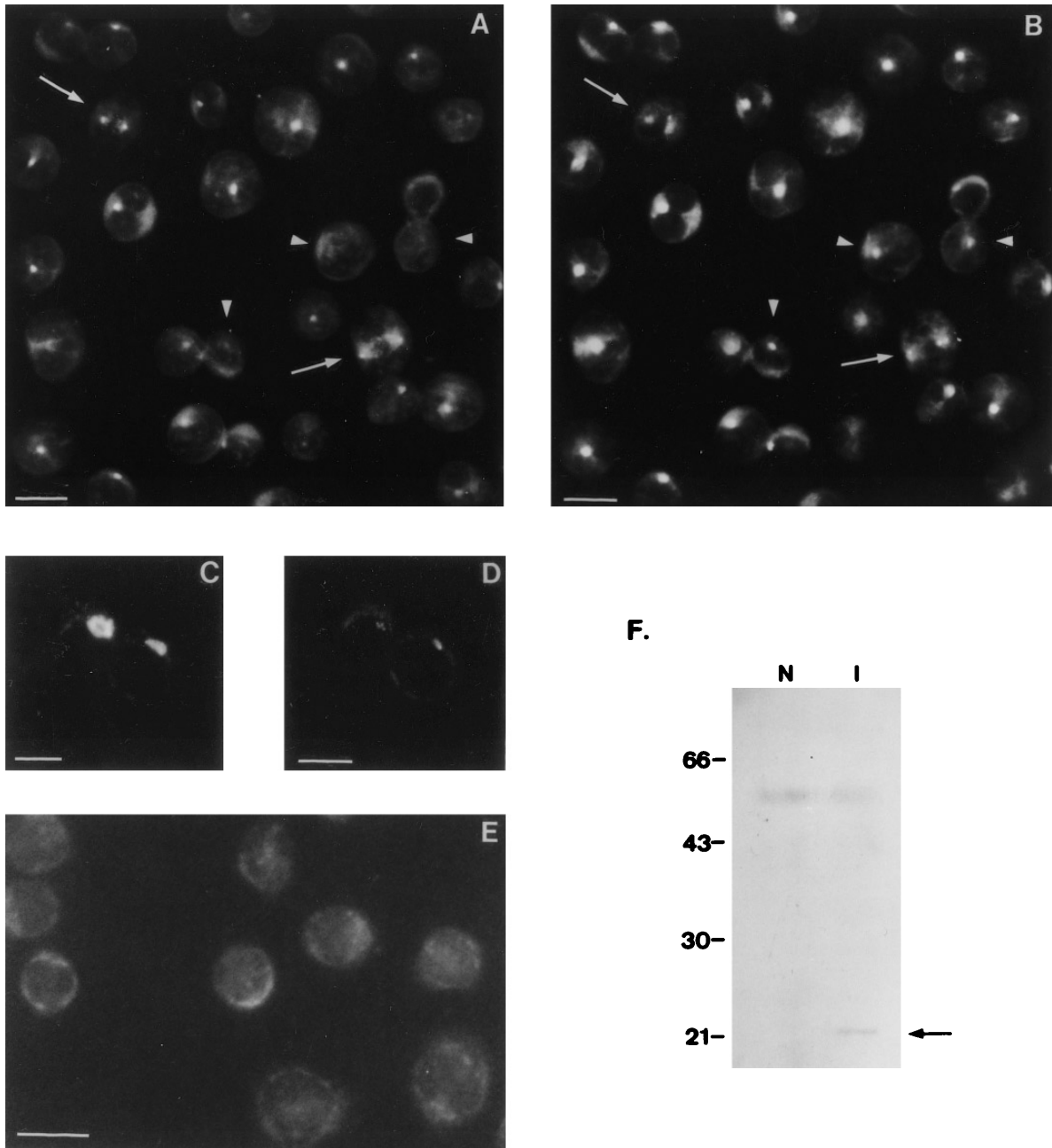


Figure 2. Sp4 14-3-3 localizes in centrosomes of FDCP leukemic cells. Acetone-fixed cells were used for double label immunofluorescence using affinity-purified rabbit anti-Sp4 (14-3-3) (2.0 μ g/ml) (A) and mouse monoclonal anti-centrin 20H5 (1:500 dilution) (B), and disclosed by fluorescein conjugated anti-rabbit (A) and Texas Red conjugated anti-mouse (B). Arrows indicate mitotic cells with duplicated centrosomes that label with anti-Sp4 and anti-centrin. Note the labeling of spindle fibers and centrosomes by anti-Sp4 (14-3-3) in the mitotic apparatus in the lower right quadrant, while anti-centrin labels only centrosomes. Arrowheads indicate cells that lack the centrosomal anti-Sp4 (14-3-3) labeling, but are labeled by anti-centrin. Cells in C and D were labeled with a Golgi-specific rabbit anti-mannosidase II (1:2000 dilution) (C) and mouse anti-centrin monoclonal antibody (D). Note that the centrosome labeling by anti-centrin lies in the center of the Golgi apparatus. The labeling of anti-Sp4 (14-3-3) is blocked by preincubation with the Sp4 peptide (E) (Bar = 10 μ m). Identification of 20 kDa centrin/caltractin (arrow) in FDCP cells by immunoprecipitation and immunoblotting (F). Lysates were immunoprecipitated with a rabbit anti-centrin IgG and immunoblotted with mouse monoclonal anti-centrin as described in “Materials and Methods”. Arrow indicates the position of centrin in lysates immunoprecipitated with immune IgG (I), but not non-immune IgG (N).

and not the Golgi apparatus because the Golgi becomes disassembled and highly vesiculated in metaphase cells (22). The specificity of the anti-Sp4 (14-3-3) labeling in the centrosome was further ascertained by 1) competing the immunofluorescent labeling of centrosomes by anti-Sp4 IgG using the Sp4 peptide and 2) verifying that FDCP cells contain a 21 kDa centrin in order to ensure that anti-centrin IgG is not detecting a cross-reacting protein (23, 24). As shown in Figure 2E, the centrosomal labeling of Sp4 (14-3-3) IgG is blocked by pre-incubation of the antibody with a 50-fold molar excess of the Sp4 peptide. In order to confirm that FDCP cells express 21 kDa centrin, detergent lysates of FDCP cells were used for immunoprecipitation with a polyclonal rabbit anti-centrin followed by immunoblot analysis of the immunoprecipitate with mouse monoclonal antibody against centrin. As shown in Figure 2F, FDCP cells contain 21 kDa centrin, thereby confirming that the immunofluorescence pattern found in cells corresponds to the expression of *bona fide* centrin/caltractin and not to an antigenically-related protein, as described in PtK₂ (M_r=165,000) (23) and KE 37 cells (M_r = 65,000–68,000) (24). The artifactual staining of the centrosomal region that is frequently observed with crude rabbit, as well as human sera is attributable to autoantibodies. We ensured that the staining observed with anti-(14-3-3) IgG was specific by 1) using only affinity-purified anti-(14-3-3) IgG and 2) using the cognate 14-3-3 peptide in a competition experiment to block staining of the centrosome.

Identification of 14-3-3 Proteins in Centrosomes Isolated by Sucrose Density Gradient Centrifugation

We next determined whether 14-3-3 proteins could be identified in centrosomes isolated from detergent lysates of FDCP cells by sucrose density gradient centrifugation. Aliquots of gradient fraction were subjected to immunoblot analysis using antibodies to α -tubulin (58 kDa) and centrin (21

kDa) to identify fractions containing centrosomes (Fig 3). Analysis of these fractions with anti-Sp4 (14-3-3) reveals a 30/32 kDa doublet (Fig. 3), indicating that the centrosome preparation contains 14-3-3 proteins. A peak of immunoreactive 14-3-3 is found in the same gradient fraction as the peak of centrin and α -tubulin. The additional 14-3-3 and centrin found in fractions of higher sucrose density may be due to aggregates of centrosomes. In order to identify the 14-3-3 isoforms present in centrosomes, a panel of anti-peptide antibodies specific for isoforms β , γ , η , σ , τ , ζ and ϵ 14-3-3 isoforms (25) was used on blots of centrosomes taken from the peak of centrin and 14-3-3 immunoreactivity. Centrosome fractions contain the γ (30 kDa) and ϵ (32 kDa) isoforms (Fig. 3A), which correspond to the 30/32 kDa doublet detected by the anti-Sp4 (14-3-3) (Fig. 1A and Fig. 3B, lane S). In order to rule out the possibility that the ϵ and γ 14-3-3 proteins are artefactually associated with centrosomes, total cell lysates of FDCP cells were analyzed with the isoform-specific antibodies. As shown in Figure 3B, FDCP cells contain four different 14-3-3 isoforms, γ , ϵ , β and ζ . However, only the γ and ϵ isoforms are present in centrosomes, but not the β and ζ isoforms. In total cell lysate, the β , ϵ , and ζ are present in equal abundance, but only the ϵ isoform is found in the centrosome fraction. This finding argues against an artifactual association of these 14-3-3 proteins with centrosomes during purification because one would expect equal or proportionate representation of the β and ζ isoforms in centrosomes as well. This finding is consistent with a specific association of the ϵ and γ isoforms with centrosomes and suggests that a subset of total cellular 14-3-3 proteins is associated with centrosomes.

Differential Localization of 14-3-3 Proteins in Centrosomes of Quiescent and Serum-Stimulated 3T3 Cells

We noted in immunofluorescence studies of asynchronously growing FDCP cells that not all

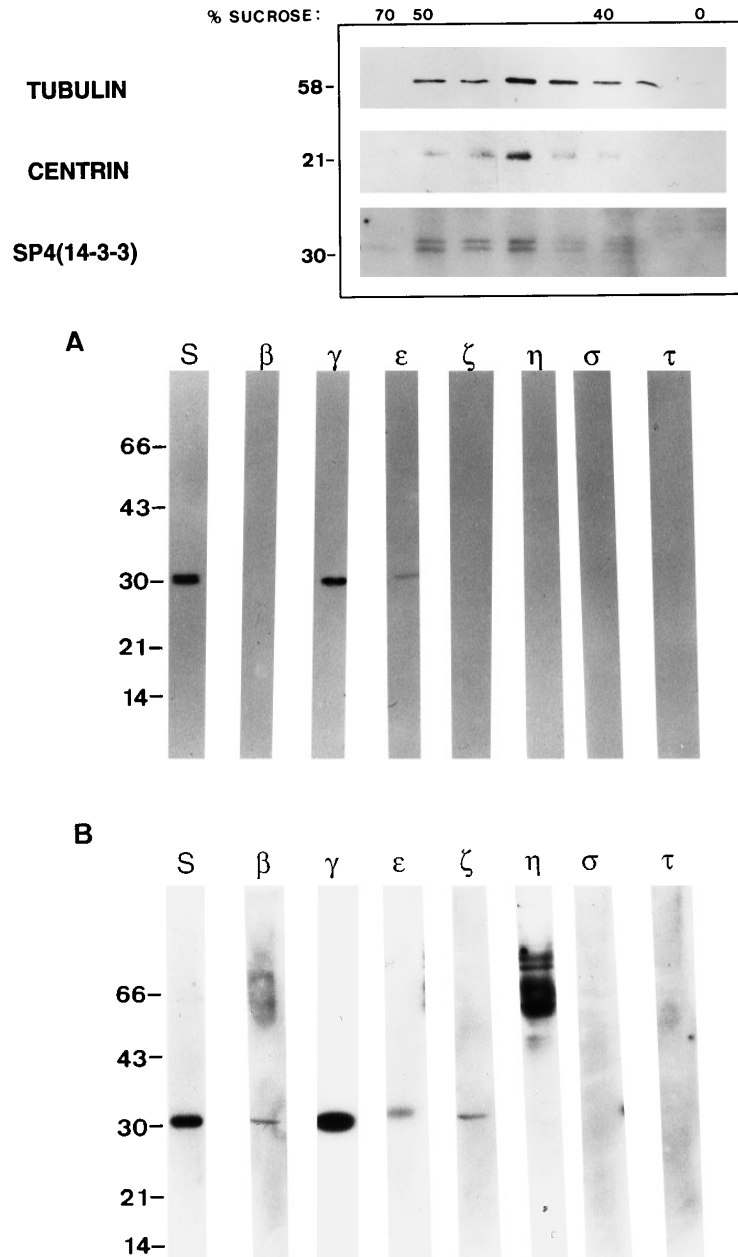


Figure 3. Centrosomes from FDCP cells contain the ϵ and γ 14-3-3 isoforms. Aliquots (100 ng protein) of sucrose gradient fractions were analyzed by SDS-PAGE and immunoblotting with anti- α -tubulin (1:1000), anti-centrin (1:2000) and affinity-purified anti-Sp4 (14-3-3) IgG (5 μ g/ml) (Upper panel). Note the doublet at 30/32 kDa disclosed by anti-Sp4 (14-3-3). A pool of centrosomes (A) banding at 45% sucrose from the sucrose density gradient and total cell lysates (B) (1 μ g/ml) were separated by SDS-PAGE and immunoblotted with 14-3-3 isoform-specific anti-peptide antibodies (1:2000 dilution, except for anti- γ which was diluted 1:5000) (Lower panel).

cells contained a discernable centrosomal signal using the Sp4 (14-3-3) antibody (Fig. 2A, arrowheads) suggesting a differential localization of centrosomal 14-3-3 proteins. In order to investigate this further, the centrosomal 14-3-3 proteins were localized in mouse 3T3 cells since these cells

are easily synchronized by serum deprivation. Furthermore, studies using 3T3 cells have shown that the duplication and separation of centrosomes during the cell cycle is controlled by serum growth factors (26-29). 3T3 cells were serum starved for 48 hr to block cells at the G₁-S border. Quiescent

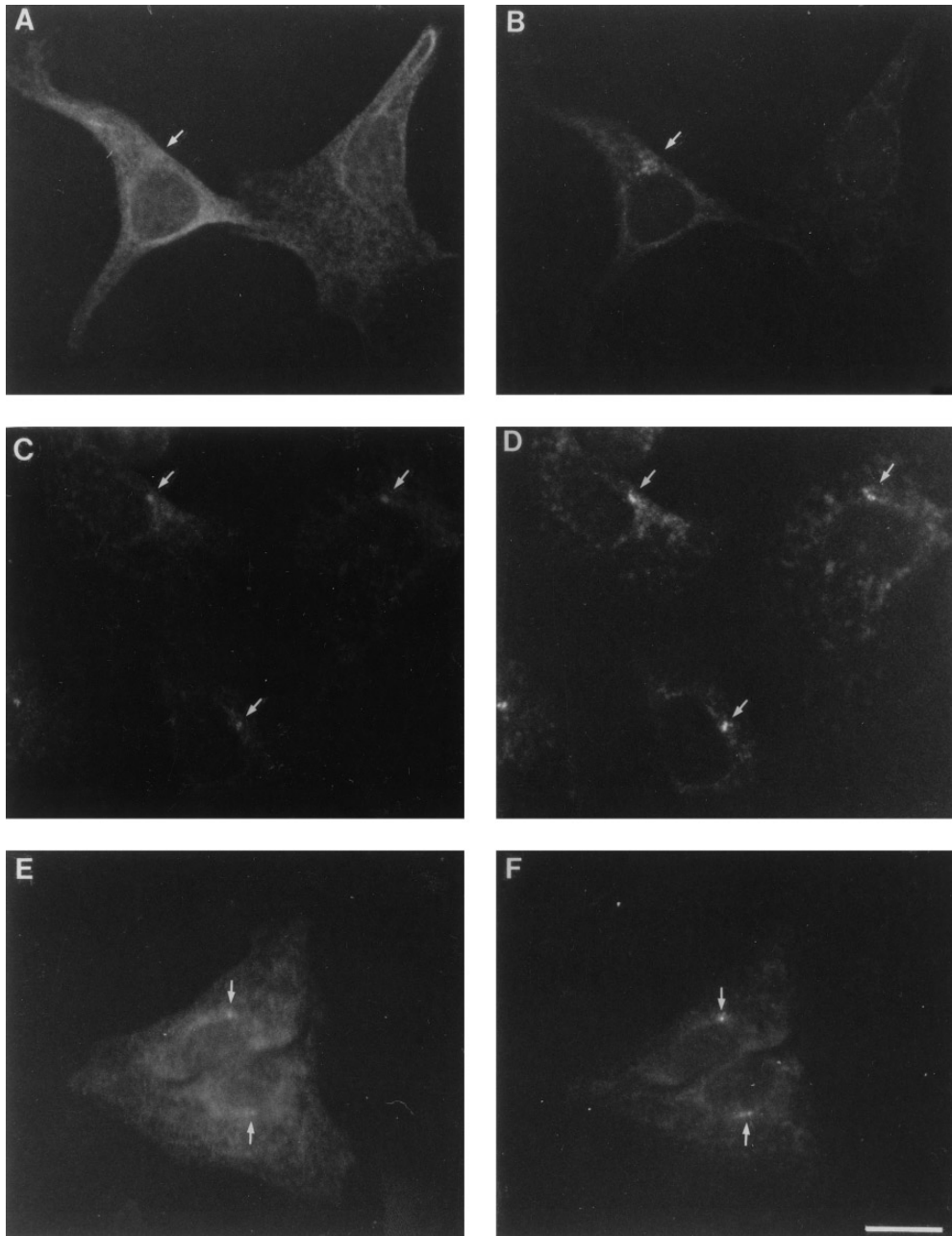


Figure 4. Differential localization of 14-3-3 proteins in the centrosome of quiescent and serum-stimulated 3T3 cells. 3T3 cells were deprived of serum for 48 hrs (A and B) followed by serum stimulation for 30 min (C and D) and 12 hr (E and F). The cells were processed for double-label immunofluorescence as described in “Materials and Methods” using anti-Sp4 (14-3-3) IgG and mouse anti-centrin monoclonal antibody. Bound anti-Sp4 IgG and anti-centrin IgG were disclosed using FITC-conjugated anti-rabbit IgG (A, C, E) and Texas Red-conjugated anti-mouse IgG (B, D, F). Bar = 10 μ m.

cells do *not* contain centrosomal 14-3-3, but label with anti-centrin indicating that quiescent cells do contain centrosomes (Fig 4A and B). Upon re-addition of serum to quiescent cells, the centrosomal 14-3-3 reappears as early as 30 min (Fig 4C and D) and is still present at 12 hr (Fig 4E and F).

DISCUSSION

In this study, we have isolated and partially sequenced 14-3-3 proteins from human spleen. Antibody raised against a 14-3-3 peptide localized both the ϵ and γ isoforms of 14-3-3 to centro-

somes; and the mitotic spindle. In studies with quiescent and serum-stimulated 3T3 cells, 14-3-3 proteins undergo a differential localization in centrosomes; in quiescent cells, the centrosomal 14-3-3 is undetectable, but reappears upon stimulation of these quiescent cells with serum. These data show that a subset of cellular 14-3-3 proteins are localized in the centrosome in response to extracellular signals and we propose that 14-3-3 proteins may play a role in signaling pathways regulating centrosome function and/or duplication during the cell cycle.

The amino acid sequence of the CNBr peptide (Sp4) isolated from human spleen is most similar to the ϵ isoform of 14-3-3 (1); this variant form, ϵ' contains a single amino acid change (T→D) at position 37 from the ϵ isoform isolated from sheep, rat, as well as human placenta and brain. HPLC analysis of human spleen 14-3-3 indicates that ϵ' and ϵ are present in nearly equimolar amounts. This variant form was also isolated from sheep brain and human placenta by reverse phase HPLC and was found to represent about 10% of the total ϵ isoform. Electrospray mass spectral analysis indicated that the ϵ isoform is 12 Da higher in mass than the ϵ' , which is consistent with a T→D change (mass difference of 14 Da) (unpublished data, A. Aitken, S. Howell and J. Madrazo). According to the recently elucidated crystal structure of 14-3-3, Thr/Asp³⁷ is located in a loop between helices B and C on the surface of the protein and is in a position to interact with other proteins (30, 31). These findings suggest that the variant ϵ' isoform represents another variant 14-3-3 present in the human spleen 14-3-3 preparation, but constitutes only 10% of the ϵ isoform in human placenta and brain. The ϵ' isoform identified here may be a tissue-specific (hematopoietic) form and its presence in placenta and brain may therefore be due to blood contamination of these tissues.

Little is known about the differential intracellular localization of 14-3-3 proteins. The only other study indicative of a differential localization of 14-3-3 was in adrenal chromaffin cells, where the γ and ϵ isoforms were found to bind to

phospholipids, while the β and ζ isoforms associated with the actin cytoskeleton (11). While the function of the serum-dependent localization of 14-3-3 proteins to the centrosome found in the present study remains speculative, it is possible that mitogenic signaling by growth factors found in serum, such as EGF and PDGF, may regulate the localization of 14-3-3 proteins to the centrosome, and perhaps to other intracellular sites as well.

It is now clear that the centrosome of animal cells plays an absolutely critical role in the cell cycle. The centrosome functions as a microtubule-organizing center (MTOC) in animal cells and undergoes duplication and separation only once during the cell cycle (see 32 for review). The duplicated centrosomes act as mitotic poles, which nucleate and organize microtubules for the formation of the mitotic spindle and the subsequent segregation of chromosomes. The centrosome of animal cells consists of a pair of centrioles each having a 9 x 3 array of microtubules and surrounding pericentriolar material (PCM). The PCM is composed of proteinaceous, electron-dense material, which is responsible for the nucleation of microtubules. However, relatively little is known about the composition and the molecular mechanisms regulating centrosome function.

A theme that is emerging for the function of 14-3-3 proteins is that they function as adaptor proteins that bring together diverse signaling molecules. The recent elucidation of the crystal structure of 14-3-3 proteins reveals that these proteins are dimeric and have two putative ligand-binding surfaces that may interact with a variety of amphipathic helices in diverse proteins (30, 31). A bewildering array of intracellular proteins have been shown to interact with 14-3-3 proteins: in neuronal cells, the interaction of 14-3-3 proteins with tryptophan hydroxylase occurs only after these enzymes are phosphorylated by Ca²⁺/CaM-dependent protein kinase II (33); 14-3-3 proteins also interact with Raf, Bcr-Abl, middle T antigen, phosphohistones and p80 cdc25 phosphatases (4, 34, 6, 35, 8). In the present paper, 14-3-3 isoforms have been localized to centrosomes and the spindle

apparatus—a major site of assembly for various cell cycle components. The inability to detect the centrosomal 14-3-3 in quiescent cells may be explained by a loss of the protein, either by degradation or translocation away from the centrosome. Alternatively, the epitope could be masked by binding other protein(s). Given the propensity of 14-3-3 proteins to interact with a wide variety of intracellular proteins, it is a strong possibility that the centrosomal 14-3-3 interacts other protein(s) that either become localized to or are integral centrosomal components that respond to mitogenic signaling. Recent studies have indicated that mitogenic signaling events may regulate centrosome duplication during the cell cycle, as well as nucleation of microtubules at the centrosome. The mitotic protein kinase, p34^{cdc2}/cyclin B (36) and cyclic AMP-dependent protein kinase A (37) have been localized to the centrosome. We and others have shown Ca²⁺/CaM-dependent protein kinase II to be present in centrosomes and the spindle apparatus by immunofluorescence and in isolated centrosomes (38, 39). Several other molecules involved in calcium signaling, including calcium, calmodulin (40) and the calcium-binding protein centrin/caltractin (41) have been localized to the centrosome and the spindle apparatus. Interestingly, centrosome duplication and separation during the cell cycle requires EGF, Ca²⁺ and calmodulin, as well as a transient activation of Ca²⁺/CaM-dependent protein kinase II (26). Thus, we propose that a possible function of centrosomal/spindle apparatus 14-3-3 may be to modulate the action of the centrosomal Ca²⁺/CaM-dependent protein kinase II. Several recent studies have shown that a transient activation of Ca²⁺/CaM-dependent protein kinase II is required for a proper G2-M transition, perhaps by regulating the ubiquitination and degradation of cyclins (42). Thus, the colocalization of 14-3-3 and Ca²⁺/CaM-dependent protein kinase II at the centrosome/spindle apparatus suggests that 14-3-3 proteins may be involved in the cell cycle, perhaps serving a function analogous to that described in

neuronal cells, where they bind to tryptophan hydroxylase after these enzymes are phosphorylated by Ca²⁺/CaM-dependent protein kinase II (33).

An alternative, or perhaps additional function for 14-3-3 proteins in the centrosome/spindle apparatus during the cell cycle may be to regulate the cdc25A and cdc25B phosphatases (8). Two members of the 14-3-3 protein family (ϵ and β) have been isolated in a yeast two-hybrid screen designed to identify proteins that interact with the human cdc25A and cdc25B phosphatases (8). While cdc25 and these 14-3-3 proteins interact *in vivo* and *in vitro*, 14-3-3 does not directly affect the phosphatase activity of cdc25A phosphatase. Furthermore, 14-3-3 proteins interact directly with Raf-1 protein kinase. While Raf-1-dependent kinase activity phosphorylates and stimulates cdc25 phosphatase activity, 14-3-3 proteins have no effect on the cdc25A-Raf-1 kinase activity. Interestingly, the inactivation of Raf-1 protein kinase by phosphatases PP1, PP2A and PTP1B has been shown to be blocked by the presence of 14-3-3 ζ (43). *In vitro* kinase activity using purified p34cdc2/cyclin B from *P. ochraceus* and histone H1 as a substrate was enhanced in the presence of histidine-tagged 14-3-3 ϵ , suggesting that the 14-3-3 protein could be preventing the dephosphorylation of histone by a phosphatase in the preparation (unpublished observations, S. Pietromonaco). Therefore, 14-3-3 may facilitate the association of cdc25 phosphatases with activated Raf-1 *in vivo*, suggesting a specific 14-3-3-mediated link between mitogenic signaling and the cell cycle at the centrosome/spindle apparatus.

Further support for the hypothesis that 14-3-3 proteins may become localized in centrosomes and the spindle apparatus in response to extracellular mitogens, comes from the recent demonstration that a significant portion of cellular MAP kinase is associated with the microtubule cytoskeleton (44). The results of the present study, in conjunction with the recent findings that 14-3-3 proteins bind to the Raf protein kinase and its

central role as a mitogenic signal transducer leading to the activation of MAP kinase lend support to a role for centrosomal/ spindle apparatus 14-3-3 (45). The “M phase-promoting factor” (MPF), which triggers the G₂/M transition of the cell cycle, is localized in the mitotic apparatus and centrosomes as an inactive complex of tyrosine-phosphorylated p34^{cdc2} and unphosphorylated cyclinB/cdc13 (46). In M phase, it is activated by the specific tyrosine dephosphorylation of the p34^{cdc2} subunit by tyrosine phosphatase p80^{cdc25}. Subcellular fractionation and immunolocalization studies have demonstrated that the mitotic kinase, p34^{cdc2} is recruited to the centrosome and the mitotic spindle during G₂/M, where it complexes with cyclin B1 (36), suggesting that the spindle apparatus is a major target for the mitotic kinase. The identification of 14-3-3 proteins with these cell cycle components at a major site of action of the kinase/phosphatase complex is consistent with a role for 14-3-3 proteins in the cell cycle.

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