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Cytoskeletal interactions of synapsin I in non-neuronal cells

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Abstract

Synapsin I is a neuronal phosphoprotein involved in the localization and stabilization of synaptic vesicles. Recently, synapsin I has been detected in several non-neuronal cell lines, but its function in these cells is unclear. To determine the localization of synapsin I in non-neuronal cells, it was transiently expressed in HeLa and NIH/3T3 cells as an enhanced green fluorescent protein fusion protein. Synapsin I-enhanced green fluorescent protein colocalized with F-actin in both cell lines, particularly with microspikes and membrane ruffles. It did not colocalize with microtubules or vimentin and it did not cause major alterations in cytoskeletal organization. Synapsin Ia-enhanced green fluorescent protein colocalized with microtubule bundles in taxol-treated HeLa cells and with F-actin spots at the plasma membrane in cells treated with cytochalasin B. It did not noticeably affect F-actin reassembly following drug removal. Synapsin Ia-enhanced green fluorescent protein remained colocalized with F-actin in cells treated with nocodazole, and it did not affect reassembly of microtubules following drug removal. These results demonstrate that synapsin I interacts with F-actin in non-neuronal cells and suggest that synapsin I may have a role in regions where actin is highly dynamic.

Keywords: Synapsin; Actin; Microtubules; Cytoskeleton; Green fluorescent protein; Membrane

Synapsins are neuron-specific phosphoproteins that associate with the cytoplasmic surface of synaptic vesicles [13,14,29,51]. The synapsin family of proteins accounts for approximately 9% of total vesicle protein and are essential in the regulation of neurotransmitter release [31,41,42]. Synapsins have also been implicated in synaptogenesis [12,17–19,33,49,53]. Overexpression of synapsin IIb in NG108-15 neuroblastoma cells leads to the appearance of synapse-like cell–cell contacts and to marked increases in the number of neuritic varicosities and in the numbers of vesicles in those varicosities [23]. Overexpression of either synapsin I or II in NG108-15 cells also enhanced functional synapse formation with myotubes [53].

Invertebrates possess a single synapsin gene, while vertebrates have at least three functional synapsin genes (I, II, and III) that likely arose from gene duplication events [30]. Synapsin I is composed of two isoforms, Ia and Ib, which are generated by alternative mRNA splicing [48]. It has an amino-terminal globular head region and an elongated, basic, glycine, and proline-rich carboxy-terminal tail [47,48].

The interaction of synapsin I with synaptic vesicles is mediated by binding of the head region to lipid components and of the tail region to protein vesicular components including CaMKII [5,6,52]. Domain B in synapsin II may also bind to protein vesicular components [51]. Domains A and C mediate binding to liposomes and peptide sequences of the C domain insert into lipid membranes [5,10,26]. Phosphorylation of the A domain of synapsin I by cAMP-dependent protein kinase and of the D domain of synapsin I by CaM kinase II (CamKII) reduced the affinity of synapsins for synaptic vesicles [26,44].

Synapsin I binds in vitro to actin [2,39], microtubules [4], brain spectrin [28,45], and neurofilaments [20,46]. Synapsin I not only binds to actin filaments ($K_d = 1$–$2\,\mu M$) but also bundles them. Actin binding and bundling are reduced upon phosphorylation by cAMP-dependent protein kinase (in domain A) and MAP kinase (at 3 sites: two in domain B and one in domain D), and are abolished by CamKII phosphorylation at two sites in the tail region.

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Recently, synapsin I has been reported in several non-neuronal cell lines [7,8,35,36]. In epithelial cells it has been localized to a *trans*-Golgi compartment [8]. Interactions of synapsin I with the cytoskeleton in non-neuronal cells have not been characterized. To determine if interactions with the cytoskeleton occur in vivo in non-neuronal cells, synapsin I was transiently expressed in HeLa and NIH/3T3 cells as an enhanced green fluorescent protein (EGFP) fusion protein. Colocalization of synapsin I with other cellular proteins was then determined.

Materials and methods

Generation of synapsin Ia-EGFP expression construct. The cDNA encoding full-length rat synapsin Ia was subcloned from pET15b-synIa (a gift from Dr. Paul Greengard, Rockefeller University, NY) into pEGFP-N1 (Clontech Laboratories, Palo Alto, CA) to generate pSynIa-EGFP. This expresses rat synapsin Ia (syn Ia) as a fusion protein with EGFP at its carboxy-terminus. The terminal aspartic acid of syn Ia is converted to a glutamic acid and the EGFP tag is preceded by a linker sequence (VPRARDPPVAT). The sequence and frame of pSynIa-EGFP were verified by automated DNA sequencing (carried out by Canadian Molecular Research Services, Ottawa, ON). The molecular weight and integrity of the fusion protein were verified by probing Western blots of total cell protein extracts from transfected cells with anti-EGFP-HRP (Clontech).

Cell culture. HeLa CCL-2 cells (ATCC, Rockville, ML) were grown in α-MEM (Invitrogen Canada, Burlington, ON) with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin/fungizone (PSF). NIH/3T3 cells (ATCC) were grown in DMEM (Invitrogen) with 10% FBS and 1% PSF. Both cell lines were grown in a 5% CO_2, 37 °C incubator.

Antibodies and probes. Alexa (350) phalloidin and rhodamine–phalloidin were from Molecular Probes (Eugene, OR) and used at 1:25 dilutions in PBS. Monoclonal mouse anti-α-tubulin 5A6 IgG was produced as described by Aitchison and Brown (1986) and used at a 1:10,000 dilution in PBS. Monoclonal mouse anti-vimentin IgG_1 clone V9 was from Sigma (Oakville, ON) and was used at 1:4000 in PBS. Primary antibodies were detected with polyclonal donkey anti-mouse-cy3 (Jackson ImmunoResearch, West Grove, PA) which had been diluted 1:400 in PBS. Hoechst 33258 was from Molecular Probes and used at 0.3 μg/ml PBS.

DNA transfection. Cells were seeded onto 22 × 22 mm glass coverslips at a density of 4×10^4 cells per coverslip. After 24 h, expression vectors (either pSynIa-EGFP or pEGFP-N1) were introduced into HeLa and NIH/3T3 cells by a calcium phosphate-mediated transient transfection method described by Chen and Okayama [11]. The cells were incubated with the transfection mix for 7 h and then rinsed with PBS and fresh growth medium was added. After incubation for 40 h, cells were fixed or treated with the appropriate drug and then fixed.

Drug treatments. Drug treatments took place at 37 °C, 5% CO_2. Treatments with nocodazole (Sigma) were for 1 h at 10 μg/ml of growth medium. Cytochalasin B (Sigma) treatments were for 45 min at 10 μg/ml of growth medium. Cells were treated with Paclitaxel (taxol; Sigma) for 7 h at a working concentration of 5 μM. Cells were fixed immediately after treatment or drug effects were reversed by rinsing cells three times with PBS, then adding fresh growth medium (without drug) and reincubating the cells for an appropriate amount of time. All drugs were stored in DMSO at −20 °C. Control cells were treated with DMSO at a final concentration equivalent to that used in drug treatments (no greater than 0.2% v/v).

Fixation and immunofluorescence/fluorescence labeling. Cells were simultaneously fixed and extracted as described by Falconer et al. [15] with slight modifications. Coverslips were rinsed briefly with PEM buffer (80 mM Pipes (pH 6.8), 10 mM EGTA, and 2 mM $MgCl_2$) followed by a 10-min fixation/extraction step in 4% paraformaldehyde/0.25% glutaraldehyde/0.5% Triton X-100 mix in PEM and finally rinsed with PBS. Free aldehyde groups were reduced by rinsing with 0.1% (w/v) $NaBH_4$ in PBS. Antibody/phalloidin incubations were for 45 min in a humidified chamber at room temperature. To visualize DNA, some samples were treated with Hoechst 33258 for 1 min. Labeled cells were examined by epifluorescence (Zeiss Axiophot microscope, Carl Zeiss, Germany) and images were digitally captured with a Hamamatsu CCD camera (Hamamatsu, Bridgewater, NJ) and MetaMorph v4.0 software (Universal Imaging, Downington, PA). Confocal images were captured using a Leitz Confocal Laser Scanning Microscope equipped with a 50 mW Kr/Ar laser. Optical sections through the *z*-axis of cells were taken at 0.5 μm

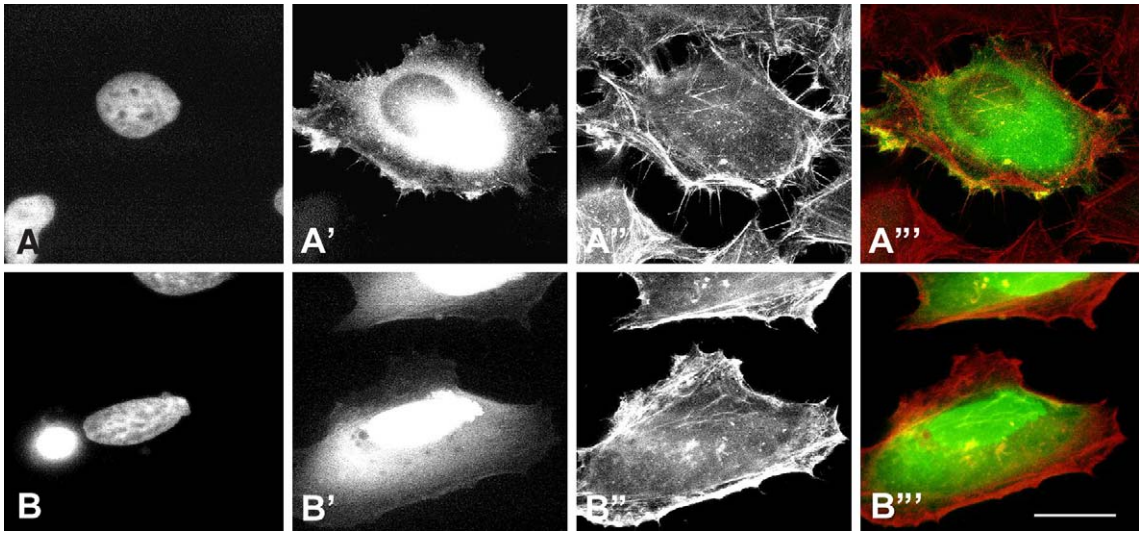

Fig. 1. Colocalization of syn Ia-EGFP with F-actin in HeLa cells as demonstrated by conventional fluorescence microscopy. HeLa cells transiently expressing either syn Ia-EGFP (A′) or EGFP (B′) were stained for F-actin using rhodamine–phalloidin (1:25 dilution; A″ and B″, respectively) and for DNA using Hoechst (A and B, respectively). Cells were simultaneously fixed and extracted with a 4% paraformaldehyde, 0.5% Triton X-100, and 0.25% glutaraldehyde solution. Images A‴ and B‴ are syn Ia-EGFP/F-actin and EGFP/F-actin overlays, respectively. Bar, 20 μm.

intervals. All images were processed using Metamorph v4.0 and Adobe Photoshop v4.0.1.

Western blotting. Transfected cells were extracted for total protein to enable verification of both the molecular weight and integrity of the expressed syn Ia-EGFP fusion protein. As positive and negative controls, total protein was extracted from cells transfected with pEGFP-N1 (vector without insert) and from untransfected cells respectively. Total cell protein in all cases was separated by SDS–PAGE and then transferred electrophoretically to nitrocellulose. Blots were probed with an anti-EGFP-horseradish peroxidase antibody–enzyme complex, and signals were detected by enhanced chemiluminescence (ECL). The syn Ia-EGFP fusion protein and EGFP (positive control) were

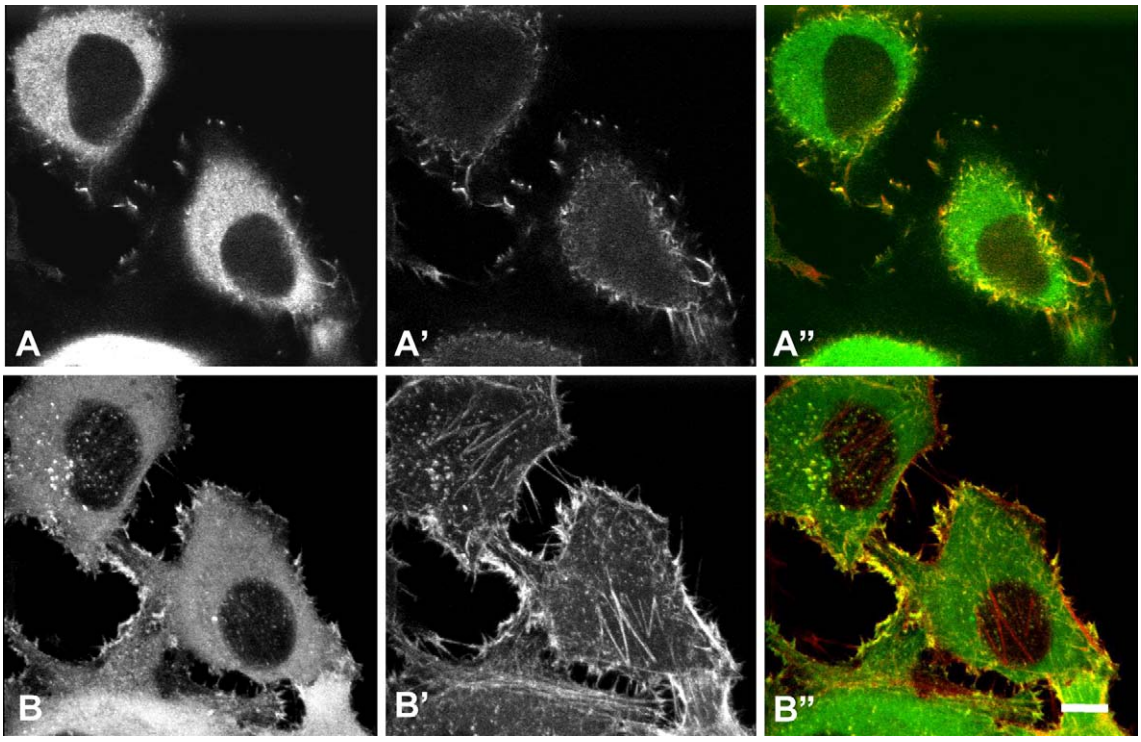

Fig. 2. Colocalization of synapsin Ia-EGFP with F-actin in HeLa cells as demonstrated by confocal laser scanning microscopy. HeLa cells transiently expressing syn Ia-EGFP (A,B) were fixed and stained for F-actin (A′ and B′, respectively). Images A-A″ are three-dimensional maximum intensity images generated from *z*-axis 0.5-μm optical sections of cells. Images B-B″ represent an optical section in the perinuclear region at the bottom of cells. Images A″ and B″ are syn Ia-EGFP/F-actin overlays. Bar, 20 μm.

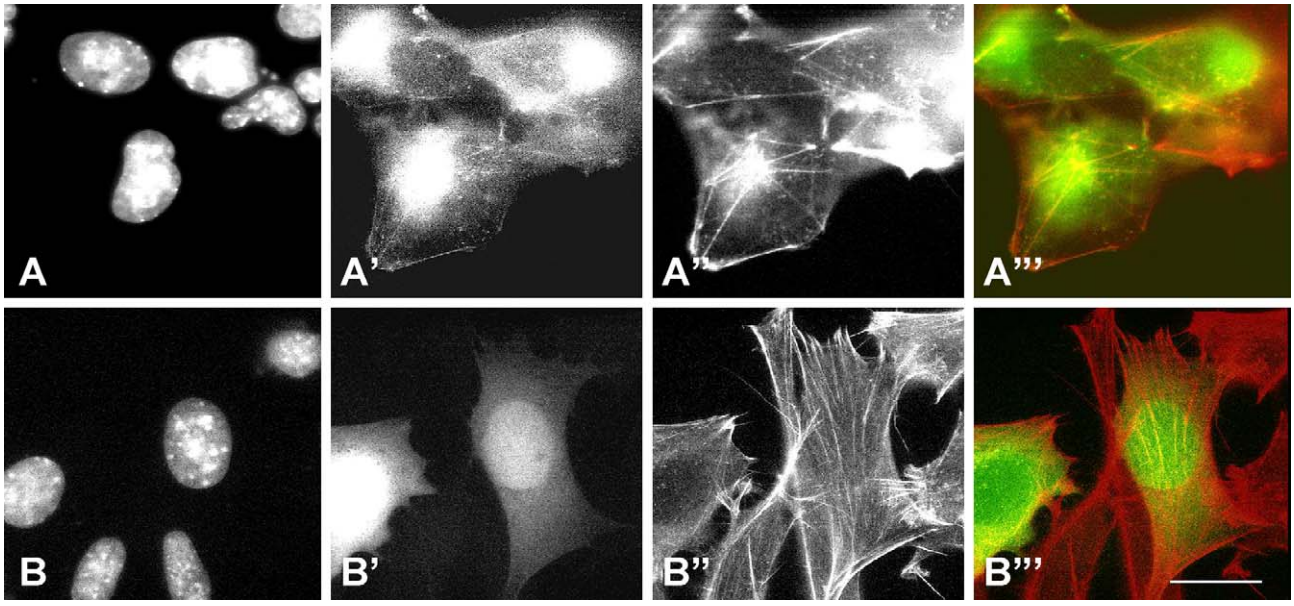

Fig. 3. Colocalization of synapsin Ia-EGFP with F-actin in NIH/3T3 cells as demonstrated by conventional fluorescence microscopy. NIH/3T3 cells transiently expressing either syn Ia-EGFP (A′) or EGFP (B′) were fixed and stained for F-actin (A″ and B″, respectively) and for DNA (A and B, respectively). Images A‴ and B‴ are syn Ia-EGFP/F-actin and EGFP/F-actin overlays, respectively. Bar, 20 μm.

expressed with the expected molecular weights (~101 and ~27 kDa, respectively) in both transfected cell lines (data not shown). No signal was detected in the negative control lanes.

Results

To determine if synapsin Ia colocalized with F-actin, we transiently expressed synapsin Ia-EGFP in HeLa cells and stained F-actin with rhodamine–phalloidin. The cells were fixed by a simultaneous fixation/extraction method [15] which extracts soluble cellular components leaving mostly insoluble components along with anything bound to these components. EGFP alone was found to be diffuse in the nucleus and cytoplasm (Fig. 1B-B‴). Synapsin Ia-EGFP was also found in the cytoplasm, especially in the perinuclear region. It was however colocalized with F-actin in microspikes and membrane ruffles (Fig. 1A-A‴).

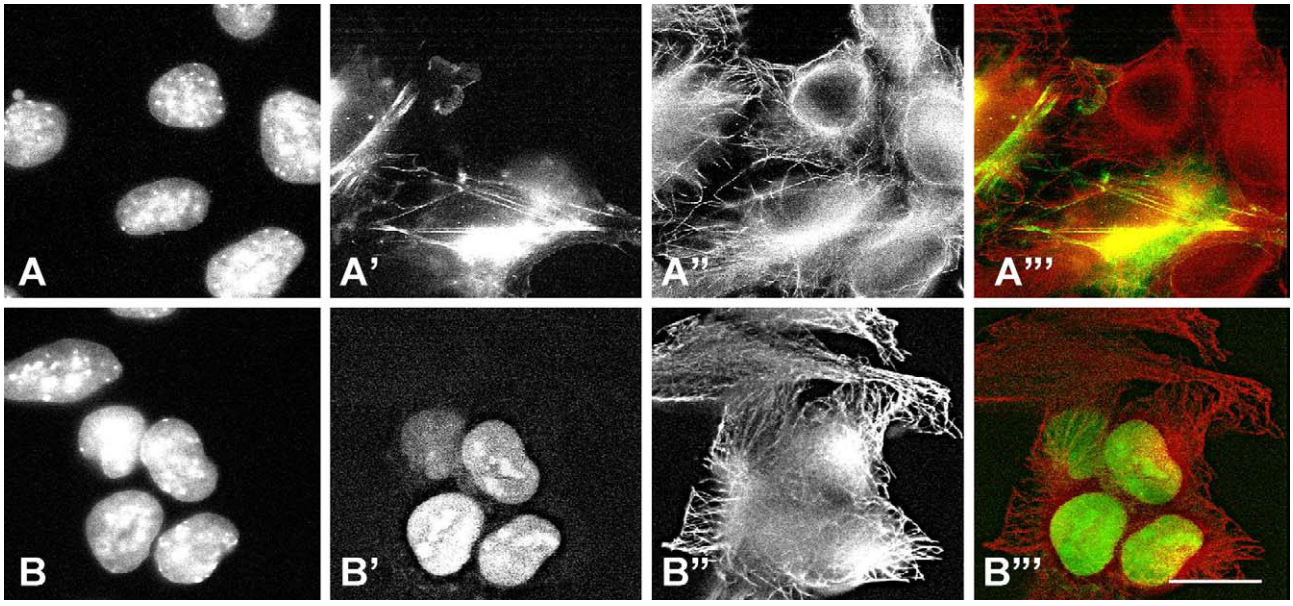

Fig. 4. Lack of colocalization of synapsin Ia-EGFP with microtubules in NIH/3T3 cells as demonstrated by conventional fluorescence microscopy. NIH/3T3 cells transiently expressing syn Ia-EGFP (A′) or EGFP (B′) were fixed and stained for DNA (A and B, respectively) and microtubules (A″ and B″, respectively). Images A‴ and B‴ are syn Ia-EGFP/microtubule and EGFP/microtubule overlays, respectively. Bar, 20 μm.

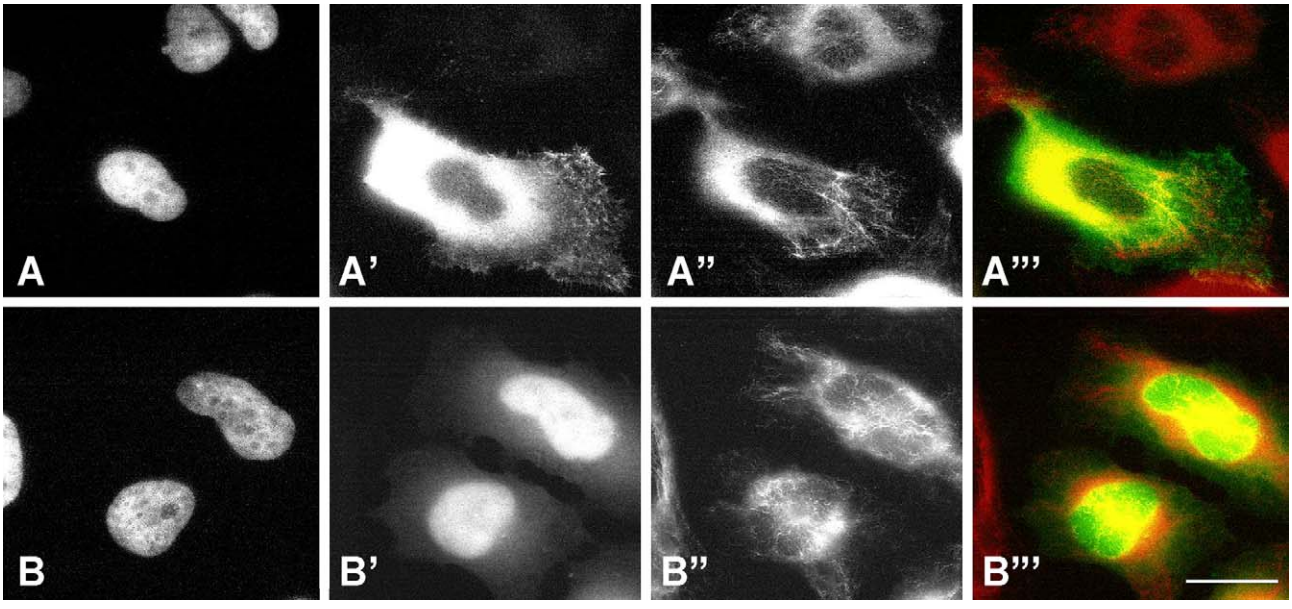

Fig. 5. Lack of colocalization of synapsin Ia-EGFP with vimentin in HeLa cells as demonstrated by conventional fluorescence microscopy. HeLa cells transiently expressing syn Ia-EGFP (A′) or EGFP (B′) were fixed as described in the legend for Fig. 7 and stained for DNA using Hoechst (A and B, respectively) and for vimentin (A″ and B″, respectively) using V9 mouse monoclonal anti-vimentin primary antibody (1:4000) and donkey anti-mouse cy3 secondary antibody (1:400). Images A‴ and B‴ are syn Ia-EGFP/vimentin and EGFP/vimentin overlays, respectively. Bar, 20 μm.

Confocal microscopy showed these results more clearly (Figs. 2A-A″ and B-B″) and also demonstrated a colocalization of synIa-EGFP with F-actin-rich spots. An optical section through the attached bottom of cells revealed that much of the syn Ia-EGFP which seemed diffusely localized to the perinuclear region was actually colocalized with F-actin (Fig. 2A-A″). No significant colocalization of syn Ia-EGFP with F-actin in the stress fibers was noted, and there was no noticeable difference in the extent of F-actin bundling.

In transfected NIH/3T3 cells, F-actin organization was not altered by syn Ia-EGFP overexpression (compare Figs. 3A″ and B″) and EGFP alone was diffuse in the nucleus and cytoplasm (Fig. 3B-B‴). However, at variance with the results obtained using HeLa cells, syn Ia-EGFP was significantly colocalized with F-actin in stress fibers in these cells (Fig. 3A-A‴). As in HeLa cells, syn Ia-EGFP also colocalized with F-actin-rich spots.

Staining of α-tubulin demonstrated that syn Ia-EGFP did not colocalize with microtubules in HeLa cells (data not shown). Overexpression of syn Ia-EGFP did not appear to alter microtubule organization nor elicit microtubule bundle formation compared to overexpression of EGFP alone (compare Fig. 4A″ with B″). When PBS was substituted for the primary antibody to control for non-specific secondary antibody binding, no signal was observed under the cy3 channel (data not shown).

HeLa cells stained for vimentin (using V9 mouse monoclonal anti-vimentin primary antibody and donkey anti-mouse-cy3 secondary antibody) demonstrated that syn Ia-EGFP did not colocalize with this intermediate filament system (Fig. 5A-A‴). Overexpression of syn Ia-EGFP did not appear to alter vimentin organization compared to overexpression of EGFP alone (compare Figs. 5A″ and B″).

When HeLa cells expressing syn Ia-EGFP or EGFP were treated for 45 min with 10 μg/ml cytochalasin B, the

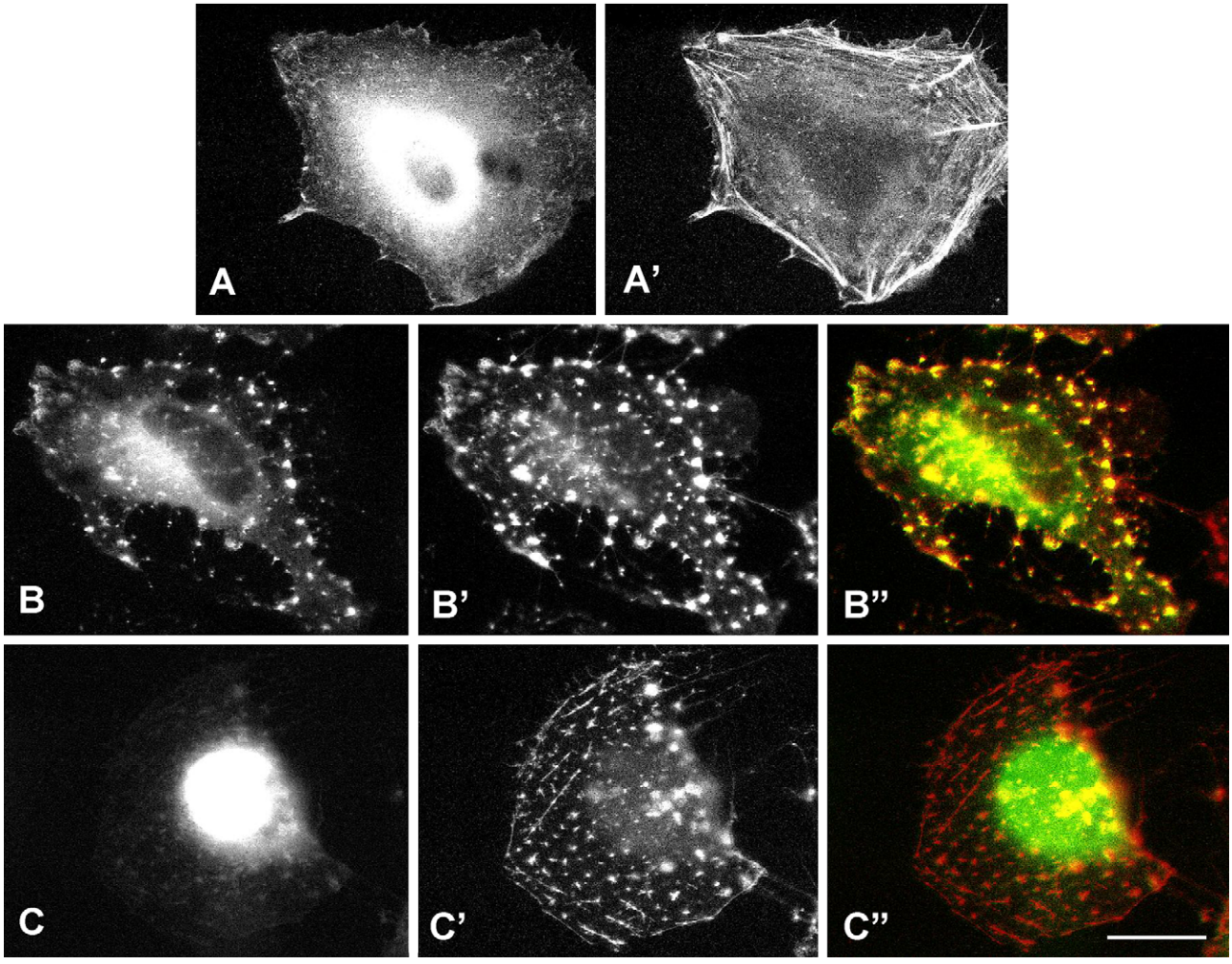

Fig. 6. Colocalization of synapsin Ia-EGFP with F-actin spots in cytochalasin B-treated HeLa cells as demonstrated by conventional fluorescence microscopy. HeLa cells transiently expressing syn Ia-EGFP (A,B) or EGFP (C) were treated with 10 μg/ml cytochalasin B for 45 min (B-B″,C-C″) or with 0.1% DMSO for 45 min (A-A′). Cells were fixed and F-actin was stained with rhodamine–phalloidin (1:25; A′–C′). B″ and C″ are syn Ia-EGFP/F-actin and EGFP/F-actin overlays, respectively. Bar, 20 μm.

F-actin fibers were no longer visible and instead F-actin was concentrated in discrete spots (Figs. 6B-B″ and C-C″, respectively). This effect was specific to the drug as demonstrated by normal F-actin distribution in control syn Ia-EGFP-expressing cells treated with 0.1% DMSO (Fig. 6A-A′). In cytochalasin B-treated cells, syn Ia-EGFP colocalized with F-actin in these spots (see overlay Fig. 6B″) whereas EGFP alone did not (see overlay Fig. 6C″).

HeLa cells expressing syn Ia-EGFP were treated as before with cytochalasin B and then the drug was removed and the cells were simultaneously fixed and extracted at 0 min (Fig. 7A-A″), 15 min (Fig. 7B-B″), 60 min (Fig. 7C-C″), and 24 h (Fig. 7D-D″) following drug removal. At each time point, syn Ia-EGFP was colocalized with F-actin in spots and in membrane ruffles (see overlays Figs. 7A″–D″). In cells undergoing cytokinesis, syn Ia-EGFP colocalized with plasma membrane-localized and cleavage furrow F-actin (data not shown). Stress fiber colocalization of syn Ia-EGFP in interphase HeLa cells was again not noted after reassembly was complete. The reassembly of F-actin over the time-course was not overtly affected by overexpression of syn Ia-EGFP (compare transfected and untransfected cells in Figs. 7A′–D′).

Discussion

In vitro studies have previously shown that synapsin I binds to several cellular components including actin and microtubules [3,6,9,16,21,22,27,37,38]. These and other observations have led to a model in which the synapsins tether synaptic vesicles to each other and to cytoskeletal components in the presynaptic nerve terminal, thereby creating a reserve pool of synaptic vesicles. This model is supported by a multitude of in vivo studies which show that synapsins are essential for the maintenance of a reserve pool of synaptic vesicles which may be used during times of high synaptic activity [25,31,32,40,42,43,49,50].

When HeLa (Figs. 1 and 2) and NIH/3T3 cells (Fig. 3) were simultaneously fixed and extracted with Triton X-100, synapsin Ia-EGFP was found to

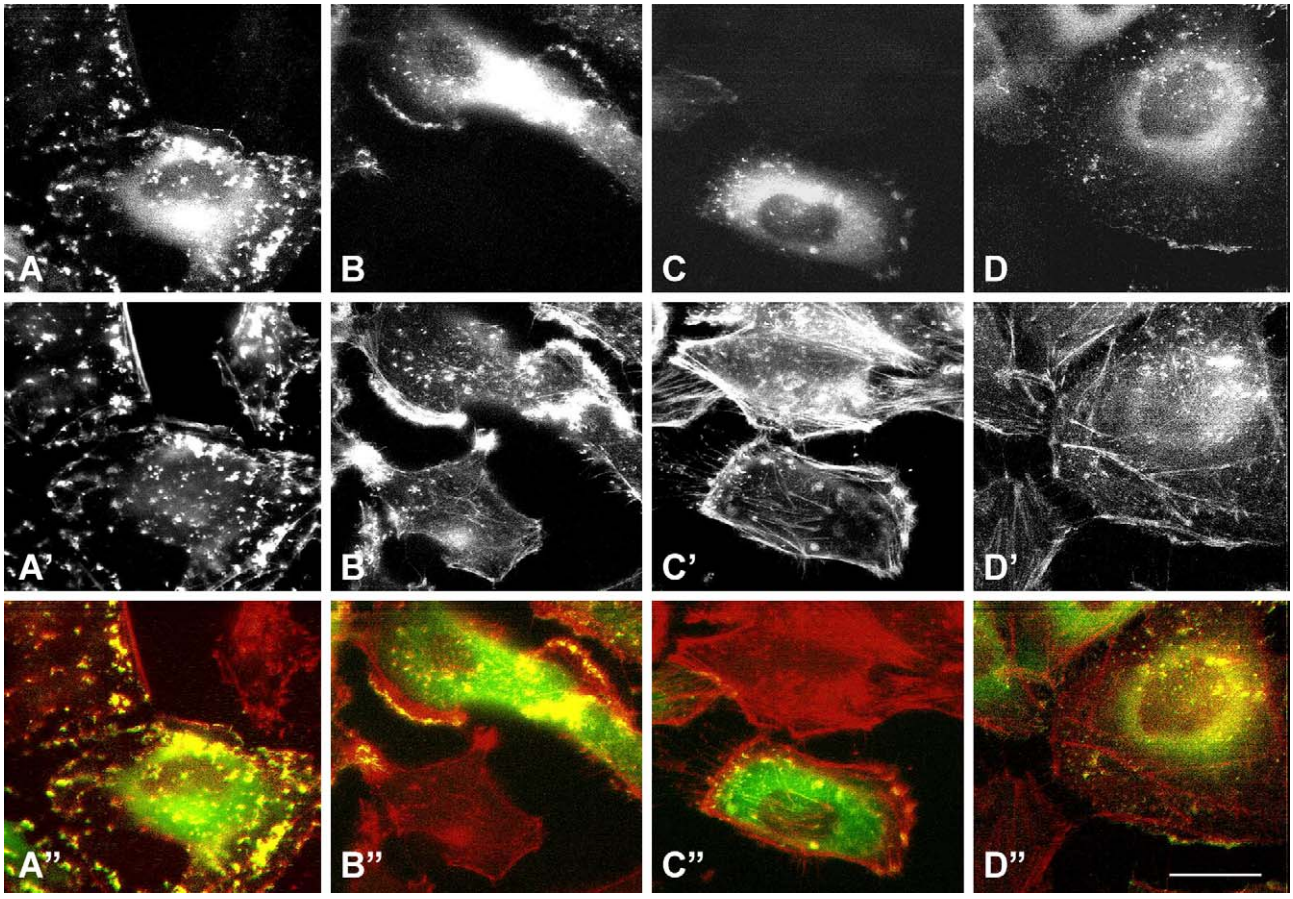

Fig. 7. Colocalization of synapsin Ia-EGFP with F-actin and lack of effect of synapsin Ia-EGFP overexpression on F-actin reassembly over a time-course of cytochalasin B wash-off in HeLa cells as demonstrated by conventional fluorescence microscopy. HeLa cells transiently expressing syn Ia-EGFP (A–D) were treated with 10 μg/ml cytochalasin B for 45 min. Cells were fixed (as described in this legend) at 0 min (A-A″), 15 min (B-B″), 60 min (C-C″) or 24 h (D-D″) following removal of the drug. F-actin was stained with rhodamine–phalloidin (1:25; A′–D′). Images A″–D″ are the respective syn Ia-EGFP/F-actin overlays. Bar, 20 μm.

colocalize with F-actin. Resistance to the extraction step, which removed soluble cellular components, suggests that synapsin I was tightly bound to F-actin.

Synapsin I did not interact in vivo with microtubules under normal cellular conditions. However, in HeLa cells treated with taxol, syn Ia-EGFP colocalized with microtubule bundles, while the EGFP tag by itself did not (data not shown). In vitro studies have shown that the affinity of synapsin I for actin is up to 5-fold higher ($K_d = 1–2\,\mu M$) than that for microtubules ($K_d = 5\,\mu M$) [24]. The taxol-induced formation of microtubule bundles simultaneously causes microtubule-bound proteins to come closer together so that they are more easily detected. It may be that the amount of synapsin Ia-EGFP per microtubule is too low to be seen in cells that have not been treated with the microtubule-stabilizing drug. However, one cannot exclude the possibility that syn Ia-EGFP binds weakly to the drug itself and not to microtubules.

Since no binding to microtubules was noted, in the absence of taxol, it may not be surprising that synapsin Ia-EGFP overexpression did not elicit microtubule bundling. It should be noted that the in vitro studies that demonstrated synapsin I-induced microtubule bundle formation used very high concentrations of synapsin I [4], and these concentrations would likely not be reached in our overexpression studies nor in neurons for that matter.

Synapsin I may also be involved in neuronal development and synaptogenesis [12,34] and has also been localized to vesicular organelles and in growth cones of regenerating axons [1]. It has been suggested that the synapsins have important roles in vesicular dynamics and may be involved in translocating vesicles to the plasma membrane in growth cones. The localization of synapsin I at actin-rich spots on the plasma membrane supports this hypothesis. In addition, our in vivo results confirm in vitro observation that synapsin I binds to F-actin, but seriously question whether in vitro binding to microtubules or vimentin occurs in vivo. Synapsins have now been identified in several non-neuronal cell types, including epithelial cells [8], osteoblasts [7], and insulinoma cells [36], however, the functions of synapsin in these cells are unclear. We have shown that synapsin I binds in vivo to actin filaments in two non-neuronal cell lines. Future studies will determine the effects of phosphorylation by various protein kinases on the actin binding activity of synapsin I in these non-neuronal cells.

Acknowledgments

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