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The association of 14-3-3γ and actin plays a role in cell division and apoptosis in astrocytes

Xiao Qian Chen[b] and Albert Cheung Hoi Yu[a,b,c,*]

[a] *Neuroscience Research Institute and Department of Neurobiology, Peking University, 38 Xue Yuan Road, Beijing 100083, China*
[b] *Department of Biology, Hong Kong University of Science and Technology, Hong Kong, China*
[c] *Hong Kong DNA Chips Limited, Hong Kong, China*

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Abstract

The 14-3-3 protein family plays critical regulatory roles in signaling pathways in cell division and apoptosis. 14-3-3γ is mainly expressed in brain. Using primary cultures of cerebral cortical astrocytes, we investigated the relationships between 14-3-3γ proteins and actin in astrocytes in cell division and under ischemia. Our results showed that endogenous 14-3-3γ proteins in immature astrocytes appeared filamentous and co-localized with filamentous actin (F-actin). During certain stages of mitosis, 14-3-3γ proteins first aggregated and then formed a ring-like structure that surrounded the daughter nuclei and enclosed the F-actin. In 4-week-old cultures of astrocytes, 14-3-3γ proteins appeared as punctate aggregates in the cytoplasm. Under ischemia, 14-3-3γ proteins formed filamentous structures and were closely associated with F-actin in surviving astrocytes. However, in apoptotic astrocytes, the intensity of immunostaining of 14-3-3γ proteins in the cytoplasm decreased. The proteins aggregated around the nucleus and dissociated from the actin filaments. Reciprocal co-immunoprecipitations demonstrated that endogenous 14-3-3γ proteins bound to detergent-soluble actin and the level of binding increased after 4 h of ischemia. As actin is a critical structural protein heavily involved in cell division and apoptotic death, our findings suggest that 14-3-3γ proteins play a role in cytoskeletal function during the process of cell division and apoptosis in astrocytes in association with actin.

Keywords: 14-3-3γ; Actin; Astrocyte; Ischemia; Cell division; Mitosis; Apoptosis

14-3-3 proteins, originally isolated in bovine brain by Moore and Perez in 1967 [1], are considered as critical regulatory and anti-apoptotic proteins in cell division and apoptosis [2,3]. In mammalian cells, seven isoforms (β, ε, γ, η, σ, τ, and ζ) of the 14-3-3 family have been identified. Previous studies indicated an isoform-specific role for 14-3-3 proteins in mammalian cells. 14-3-3 isoforms have distinct tissue and subcellular localizations [4–6]. For example, the σ isoform was mainly expressed in epithelial cells [4], whereas the γ isoform was mainly expressed in brain [1,5]. In sheep brain, only γ, ε, and η but not β and ζ were associated with synaptic junctions [6]. Isoforms of 14-3-3 also have distinct binding partners [7,8]. For example, complex formation between PKC-ζ and Raf-1 was mediated strongly by 14-3-3β but not by ζ [7]. In the brain, 14-3-3γ was previously thought to be neuronal specific [5] and was selectively elevated in Alzheimer's disease, Down Syndrome [9], and Creutzfeldt–Jakob disease [10]. We have recently found that 14-3-3γ was also located in astrocytes and both its gene and protein were up-regulated under ischemia (submitted for publication). This suggests a critical physiological or pathological role for 14-3-3γ in the brain.

Recent evidence indicates that 14-3-3 proteins might play a critical role in regulating cellular activities by associating with cytoskeletal proteins [11–13]. For example, 14-3-3ζ and σ bind to phosphorylated keratin [11]. This association might limit the binding of 14-3-3 to cdc25, causing cdc25 to enter the nucleus to activate cdc2 and promote mitosis [2,3]. Incubation of bovine adrenal chromaffin cells with purified 14-3-3 proteins in the presence of MgATP would alter the structure of F-actin [12]. In budding yeast, the expression of dominant-negative alleles of 14-3-3 proteins resulted in the clustering of actin molecules [13]. These results indicate that

* Corresponding author. Fax: +852-2111-9762.
E-mail address: achy@dnachip.com.hk (A.C.H. Yu).

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14-3-3 proteins associate with actin and regulate its organization. However, in these studies the expression of 14-3-3 was manipulated artificially. The interaction of 14-3-3 and cytoskeletal proteins including actin under physiological conditions has not been studied previously. In this study, we investigated whether endogenous 14-3-3γ could interact with actin to regulate cellular function.

Actin is a major cytoskeletal protein that controls cellular structure and the processes of cell division [14] and apoptosis [15–17]. Reorganization of actin is required for cell division [10]. Subjected to ischemic insults, actin might reassemble or dissociate due to hyper-phosphorylation or dephosphorylation [16–18]. Previous studies indicated that actin might be regulated by various kinases such as Raf [19], PKC [20], and MAP kinase [21]. Moreover, actin is a substrate of activated caspases, the executors of apoptosis [18]. The disruption of actin results in progression to apoptosis under ischemia [15–17]. This indicates that the regulation and organization of actin are complicated processes involved in the control of cell division and apoptosis.

In this study, we demonstrated that endogenous 14-3-3γ proteins are associated with both filamentous-actin (F-actin) and soluble actin in astrocytes during cell division and under ischemia. This suggests that the association between 14-3-3γ and actin during cell division and apoptosis under ischemia in astrocytes has a critical physiological role.

Materials and methods

Primary cultures of cerebral cortical astrocytes. The cultures were prepared from the cerebral cortex of newborn ICR mice (Animal Care Centre, HKUST) as reported previously [22,23]. For each 35- or 100-mm Falcon tissue culture dish (Becton–Dickinson, USA), 2 or 10 ml cell suspension was plated, respectively. All cultures were incubated in a Napco CO_2 incubator (Precision Scientific, USA) at 37 °C, air:CO_2 (95:5) (v/v) and 95% humidity. The culture medium was changed 2 days after seeding and, subsequently, twice per week with DMEM containing 10% (v/v) FCS for the first 2 weeks and 7% (v/v) thereafter. Cultures were not used for ischemia experiments until they were at least 4 weeks old.

Anaerobic chamber-induced ischemia model. The use of anaerobic chambers to induce ischemia has been reported in various studies [22,23]. Briefly, ischemia media (free of glucose and serum) were degassed for 30 min with N_2 and re-gassed with N_2:CO_2:H_2 (85:5:10) for 20 min before use. The ischemia media and cultures were then transferred into an anaerobic chamber (Forma Scientific, USA) saturated with N_2:CO_2:H_2 (85:5:10). The oxygen concentration in the ischemia media and the anaerobic chamber was 0.1 ppm as measured with a dissolved oxygen meter (Hanna Instruments HI9142, Italy) and 0.1% (v/v) as detected by an MSA gas meter (Passport Personal Alarm, USA), respectively. The astrocyte cultures were washed three times with ischemia media after which the cells were covered with 6.4 ml ischemia media for 100-mm dishes or 0.78 ml for 35-mm dishes. All culture dishes were wrapped with Parafilm to prevent evaporation during incubation. Normal cultures (0 h ischemia) were used as controls.

Immunostaining. Immunostaining was performed at room temperature (RT). Before staining, cultures of astrocytes were washed twice with PBS. The cells were fixed with 4% paraformaldehyde for 15 min. After washing twice with PBS, the cells were permeabilized with 0.2% Triton X-100 for 15 min and then blocked with 3% bovine serum albumin for 2 h. After incubation with 14-3-3γ antibodies (C-16, Santa Cruz Biotech, USA) for 2 h, the cells were washed three times with PBS and incubated with FITC-conjugated secondary antibodies for 1 h. After extensive washing, rhodamine phalloidin (Sigma, USA) diluted 1:200 was used to stain actin for 10 min. Hoechst 33342 (2 μg/ml) was used to stain the nucleus for 5 min. Micrographs were taken with a Leica DMR fluorescent microscope (Germany) or a laser scanning confocal microscope (Bio-Rad MRC-600, USA).

Co-immunoprecipitation (co-IP) and Western blot analysis. Cell lysates were prepared by lysing cells in ice-cold lysis buffer (150 mM NaCl, 0.1% (w/v) Triton X-100, 20 mM Tris (pH 7.6), 0.1 mM phenylmethylsulfonyl fluoride, 0.7 μg/ml leupeptin, and 0.5 μg/ml pepstatin). Four hundred micrograms of total soluble protein (cell lysate) from the astrocyte culture was used in co-IP. Lysates were pre-cleared for 3 h at 4 °C using 10 μl protein A–agarose with minor agitation. After centrifugation at 3000 rpm, supernatants were incubated with 4 μg of 14-3-3γ or actin antibodies (Santa Cruz Biotech, USA) at 4 °C overnight. Twenty microlitres of protein A–agarose was added and incubated for another 3 h as previously described. After centrifugation, supernatants were discarded and the precipitate was washed three times with lysis buffer. After final centrifugation, 20 μl protein loading buffer was added to the precipitate and boiled. Supernatants were subjected to Western blot analysis with the 14-3-3γ or actin antibodies as described above. Normal rabbit immunoglobin G (IgG) was used as the negative control for co-IP. Boiled proteins were resolved on a 12% SDS–PAGE mini-gel under reducing conditions. Ten micrograms of cell lysate protein was used as controls for Western blot analysis. They were then electrophoretically transferred onto nitrocellulose membranes. The membranes were blocked with 5% (w/v) non-fat dried milk in TBST buffer (0.1 M Tris–HCl, pH 8.0, 0.9% (w/v) NaCl, and 0.1% (v/v) Tween 20) and probed with diluted polyclonal antibodies to 14-3-3γ and actin, in TBST for 2 h at RT. After incubation with corresponding HRP-conjugated secondary antibodies (1/4000; Amersham, France) for 1 h at RT, antigens were revealed by chemiluminescence (Amersham, France).

Results

Co-localization of 14-3-3γ proteins with F-actin in young astrocytes

Astrocytes grown in primary cultures proliferated mainly between days 7–14. In the first 3 days after seeding, astrocytes were recovering from the trauma created by dissection and separation from other brain tissues. After day 7, astrocytes began to proliferate actively and cells at various stages of division could be observed. After day 14, cultures began to reach confluence and the number of dividing astrocytes drastically decreased.

The distribution of endogenous 14-3-3γ proteins and their relationship with F-actin in 7-day-old astrocytes was visualized by co-immunostaining with 14-3-3γ antibodies and rhodamine phalloidin (Fig. 1). The filaments of cytoskeleton in the astrocytes (indicated by arrows) were evident under phase contrast and fluorescent microscopy. A condensed structure was apparent (indicated by a large arrowhead) through which several filaments crossed. Staining of 14-3-3γ proteins and actin showed a filamentous distribution throughout the cytoplasm similar to that of elements of the cytoskeleton as revealed under phase contrast and fluorescent

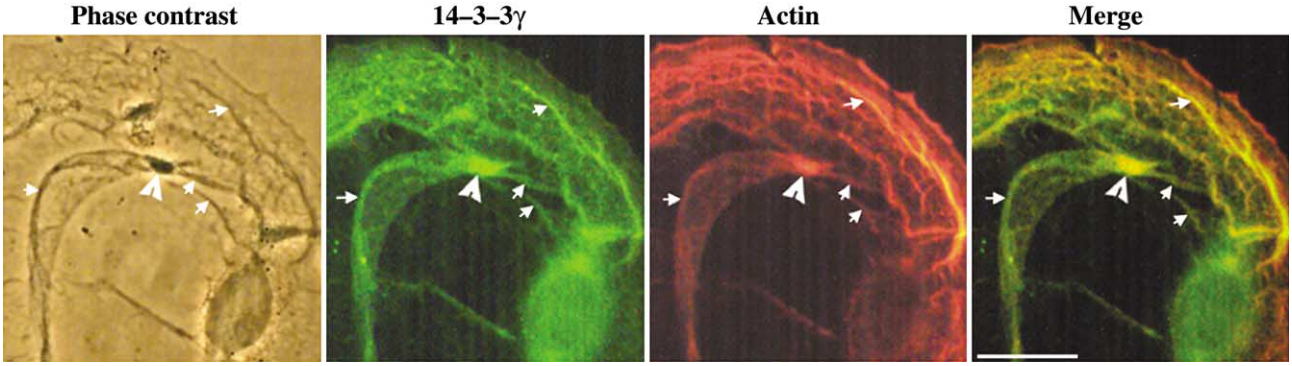

Fig. 1. Co-localization of 14-3-3γ proteins and actin in astrocytes. Primary cultures of astrocytes at day 7 were stained with 14-3-3γ antibody and rhodamine phalloidin simultaneously. Micrographs are phase contrast, staining of 14-3-3γ (green), actin (red), and the merged image of the same fields. Arrows indicate fibers of 14-3-3γ proteins or actin. Large arrowheads indicate a condensed structure. Co-localization of 14-3-3γ fibers with those of actin appear yellowish in the merged image. Bar, 20 μm.

microscopy (indicated by small arrows). At the site of the large condensed structure, both 14-3-3γ and actin were also highly condensed (indicated by a large arrowhead). The merged image clearly showed that the filaments of 14-3-3γ proteins co-localized with those of actin (yellowish in merged image).

Distribution of 14-3-3γ and its interaction with actin during mitosis

The distribution of 14-3-3γ proteins and their association with F-actin were followed in astrocytes during mitosis (Fig. 2). It is known that F-actin has to be disassembled for mitotic progression [14]. As shown in the phase contrast and actin-specific staining images, the filamentous structures of the cytoskeleton in the astrocytes in Fig. 2 were not as evident as those in the astrocytes in Fig. 1, indicating that the cells in Fig. 2 were undergoing mitosis. Astrocytes in (B) and (C) are clearly in the later stages of mitosis as the two daughter nuclei are seen to be separating. In (A), a condensed structure was found near the nucleus (indicated by an arrow) under phase contrast and fluorescent microscopy. At this location, a large aggregate of 14-3-3γ proteins was

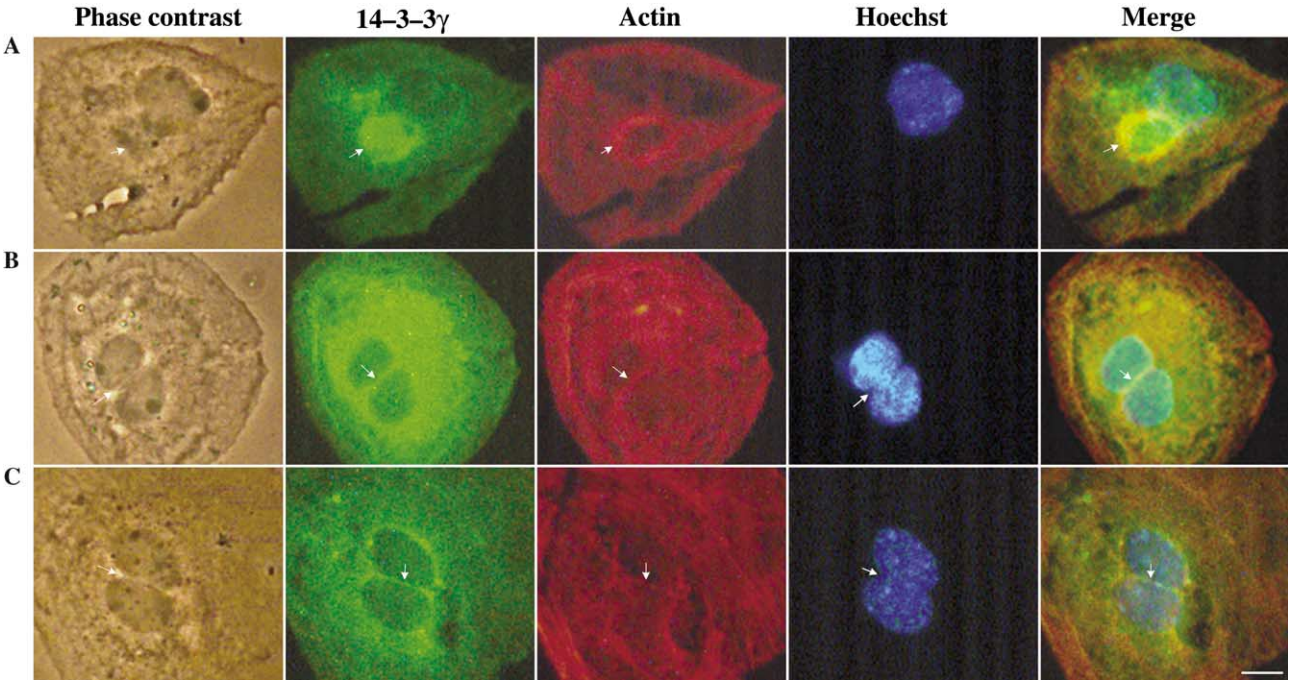

Fig. 2. Distribution of 14-3-3γ proteins and their association with actin during mitosis in astrocytes. Primary cultures of astrocytes were stained with 14-3-3γ antibodies, rhodamine phalloidin, and Hoechst 33342 simultaneously. Three individual astrocytes (A–C) are shown. (A) 14-3-3γ proteins aggregate before nuclear division occurs. (B) Daughter nuclei are surrounded by 14-3-3γ proteins during nuclear division. Filamentous actin is co-localized in the 14-3-3γ clusters or rings. Bar, 20 μm.

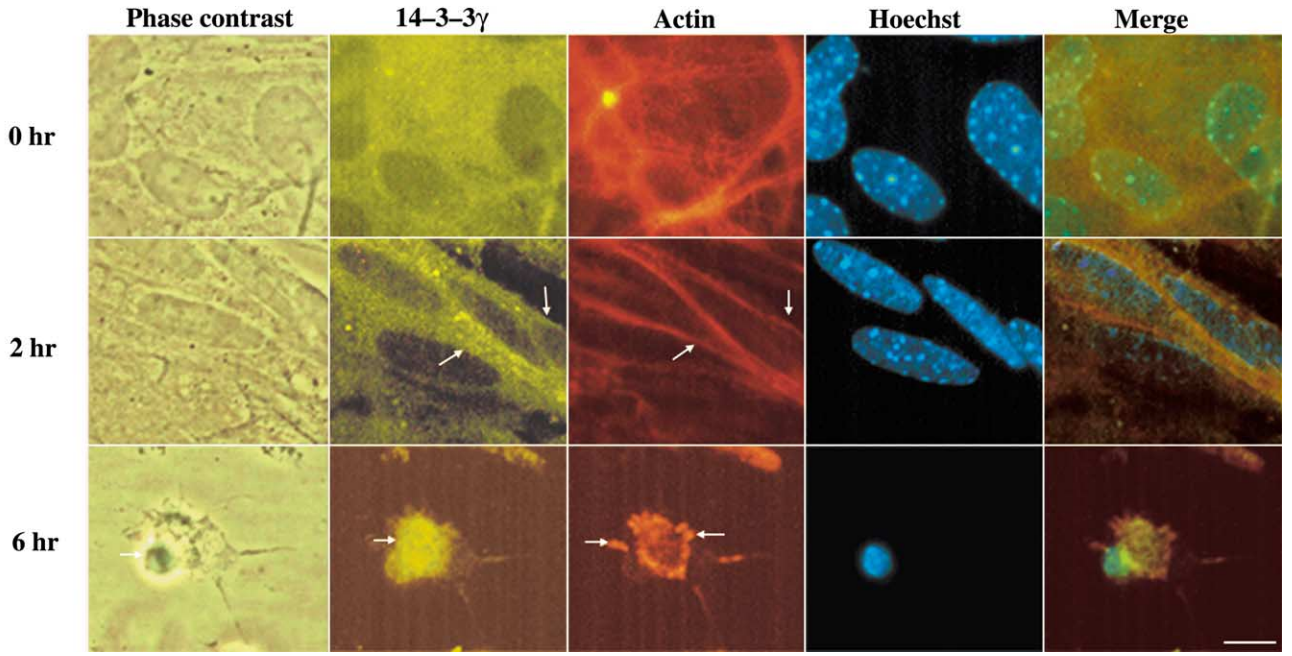

Fig. 3. Association of 14-3-3γ proteins with actin under ischemia. Primary cultures of astrocytes at day 28 were used for ischemia studies. Immunostaining was performed as previously described. 14-3-3γ fibers in mature astrocytes before ischemia treatment (0 h) appear as fine dots. After 2 h of ischemia, 14-3-3γ filaments are evident (indicated by arrows). Thinner fibers of actin are localized inside the 14-3-3γ filaments (indicated by arrows). After 6 h of ischemia, 14-3-3γ aggregates in the apoptotic astrocyte and the actin fibers become disorganized. Actin also dissociates from 14-3-3γ and becomes aggregated as dot-like structures (indicated by arrows). Bar, 20 μm.

evident surrounded by a shell of F-actin. In the astrocyte in (B), a dense assembly of 14-3-3γ surrounded the two separating daughter nuclei. A thin fiber of F-actin was visible between the separating daughter nuclei (indicated by an arrow). The merged image showed that the actin fiber coincided with the 14-3-3γ proteins (indicated by an arrow). The astrocyte shown in (C) is at a later stage of mitosis than that shown in (B), as the chromosomes in the two daughter nuclei are clearly visible as evenly distributed spots with Hoechst staining. These chromosome spots were also found in all mature astrocytes by day 28 (Fig. 3, Hoechst staining). Fewer 14-3-3γ proteins were seen surrounding the daughter nuclei (indicated by an arrow) in (C). The actin fiber between the two daughter nuclei was still evident and co-localized with the 14-3-3γ filaments (indicated by an arrow).

Interaction of 14-3-3γ with actin under ischemia

The association of 14-3-3γ proteins with F-actin in astrocytes at day 28 under normal and ischemic conditions was studied using co-immunostaining (Fig. 3). Under normal conditions (0 h ischemia), 14-3-3γ proteins were mainly distributed in these astrocytes as fine punctate structures in the cytoplasm whereas actin appeared as filamentous structures. After 2 h of ischemic incubation, the 14-3-3γ proteins appeared as filaments (indicated by arrows). Very fine F-actin filaments (indicated by arrows) clearly co-localized with the 14-3-3γ protein filaments. After 6 h of ischemia, some astrocytes showed apoptotic characteristics under phase contrast microscopy and by Hoechst staining. In the apoptotic astrocytes, 14-3-3γ proteins aggregated around the nucleus (indicated by arrow) and the fine filaments of actin became disorganized. The actin dissociated from 14-3-3γ and formed aggregates (indicated by arrows).

The binding of endogenous 14-3-3γ proteins and soluble actin in astrocytes was confirmed by a reciprocal co-IP of 14-3-3γ and actin (Fig. 4). IP of cell lysates using actin antibodies, and subsequent Western blotting with antibodies specific to 14-3-3γ proteins, indicated that actin bound to 14-3-3γ (Fig. 4A). The cell lysate, as the positive control for Western blot analysis, confirmed that the band was 14-3-3γ protein. The negative control (co-IP with normal IgG) confirmed that the binding between actin and 14-3-3γ proteins was specific. The binding of actin to 14-3-3γ was also verified by reciprocal co-IP using antibodies to 14-3-3γ (Fig. 4B). After 4 h of ischemia, more 14-3-3γ proteins were found to bind to actin in astrocytes.

Distribution of 14-3-3γ in apoptotic astrocytes under ischemia

The distribution of 14-3-3γ proteins in individual astrocytes after 6 h of ischemic incubation was studied further with both fluorescent (Fig. 5A) and confocal microscopy (Fig. 5B). In the surviving astrocytes,

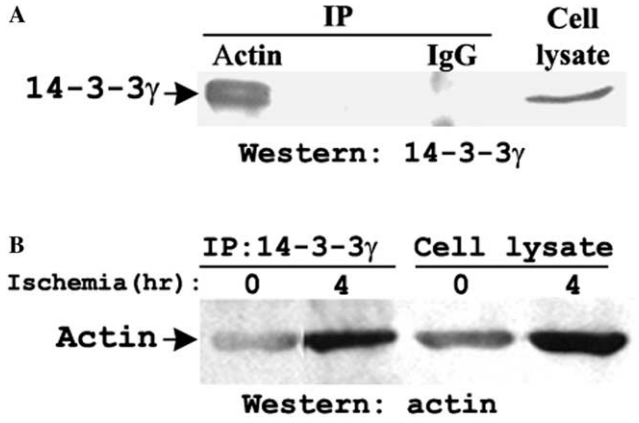

Fig. 4. Binding of endogenous 14-3-3γ and soluble actin in normal astrocytes (A and B) or astrocytes under 4 h of ischemia (B). Four micrograms of actin antibodies (A), normal rabbit IgG (A), or 14-3-3γ antibodies (B) was used for co-IP with 400 μg total soluble astrocyte lysate proteins. IP treatment with normal rabbit IgG was used as the negative control. Antibodies to 14-3-3γ were used in Western blot analysis (A) to detect 14-3-3γ proteins co-precipitated with actin. No 14-3-3γ was found to co-precipitate with normal IgG. Antibodies to actin were used in Western blot analysis (B) to detect actin co-precipitated with 14-3-3γ. After 4 h of ischemia, more actin co-precipitated with 14-3-3γ as shown by the intense bands. Cell lysates containing 20 μg total soluble proteins without IP treatment were used as positive controls (A and B).

filaments of cytoskeleton were clearly seen under phase contrast microscopy (indicated by arrows, Fig. 5Aa). 14-3-3γ protein filaments were also evident (indicated by arrows, Fig. 5Ab) and were distributed in a similar pattern as the cytoskeleton filaments. In apoptotic astrocytes, the cytoskeletal filaments were disorganized (Fig. 5Ad) and the 14-3-3γ proteins formed aggregates around the nuclei (indicated by arrows, Fig. 5Ae). Confocal microscopy (Fig. 5B) indicated that 14-3-3γ proteins were mainly distributed in astrocytes as fine dot-like structures, which decreased in density near the cytoplasmic membranes (indicated by arrows, Fig. 5Ba). In the astrocyte shown in Fig. 5Bb, aggregates of 14-3-3γ proteins (indicated by arrows) were evident. In Fig. 5Bc, 14-3-3γ protein aggregates (indicated by arrows) were concentrated around the nucleus.

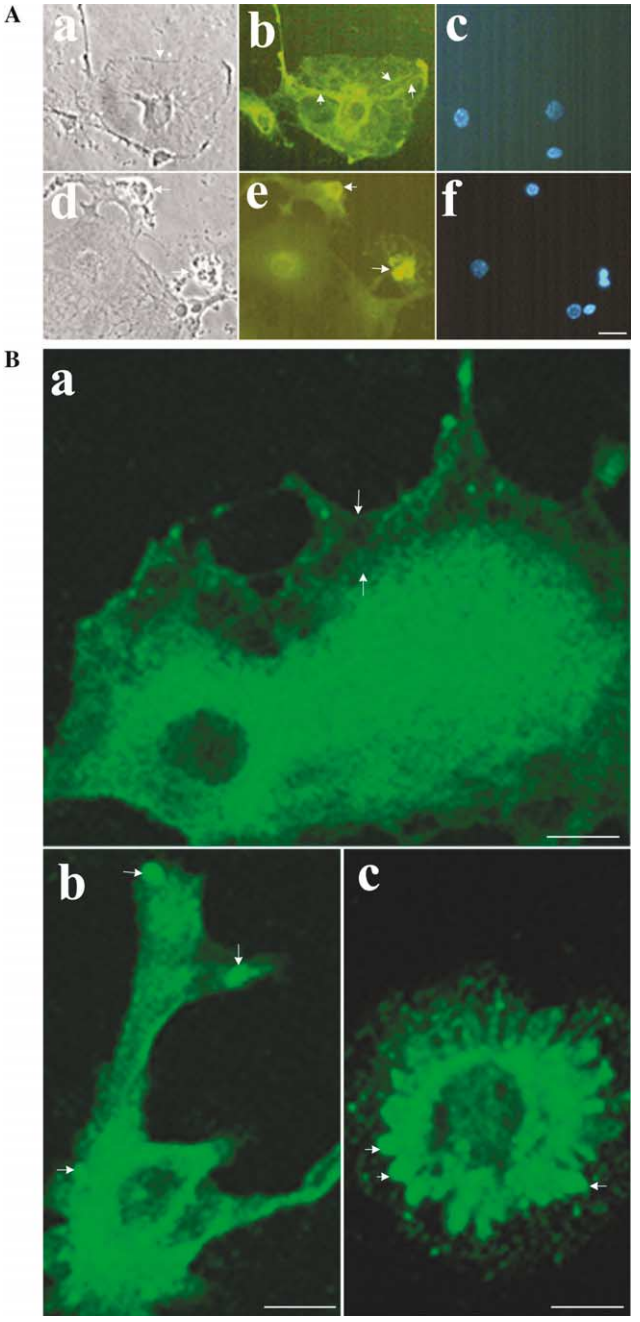

Fig. 5. Distribution of 14-3-3γ proteins and the organization of the cytoskeleton in surviving and apoptotic astrocytes under ischemia. Distribution of 14-3-3γ proteins in individual astrocytes after 6 h of ischemia was studied with a fluorescent (A) and confocal microscope (B), respectively. Upper and lower panels in (A) show micrographs of the same field under phase contrast (a, d), 14-3-3γ staining (b, e), and Hoechst staining (c, f). Cytoskeletal and 14-3-3γ fibers in the surviving astrocytes (Aa and Ab, indicated by arrow) are evident. In dying or apoptotic astrocytes (Ad–f), the concentration of 14-3-3γ decreased in the cytoplasm and increased around the nuclei (Ae, indicated by arrows). The decrease in the concentration of 14-3-3γ proteins in the cytoplasm and the aggregation of these proteins around the nucleus in ischemic astrocytes was also demonstrated under confocal microscopy (B, indicated by arrows). Bars, 20 μm.

Discussion

The 14-3-3 protein family contains soluble proteins that are found mainly in the cytoplasm. Information about the structure and distribution of 14-3-3 isoforms in mammalian cells is scarce. In this study, we found that endogenous 14-3-3γ proteins in astrocytes appeared as distinct structures during mitosis and after ischemic insult. In 4-week-old normal astrocytes, 14-3-3γ proteins were located mainly in the cytoplasm and distributed as fine dot-like structures, consistent with the hypothesis that 14-3-3 proteins exert their primary physiological

function in the cytoplasm [2,3]. Clusters or ring-like structures of 14-3-3γ proteins around the nuclei were found in astrocytes undergoing mitosis. 14-3-3γ proteins also formed filamentous structures in astrocytes that survived ischemic insult. In apoptotic astrocytes generated after ischemic incubation, prominent aggregates of 14-3-3γ proteins around the nucleus were observed. These new findings suggest that the structure and cellular distribution of 14-3-3γ proteins are related to some physiological processes such as cell division and apoptosis.

It is known that several isoforms of 14-3-3 proteins, such as 14-3-3σ and ζ, play critical roles in regulating the cell cycle [3,11]. To date, it is still unclear whether the γ isoform is also involved in cell division. In this study, we found that 14-3-3γ proteins clustered around the dividing nuclei. These distinct clusters were observed only when the two daughter nuclei appeared clearly during mitosis. This unique distribution of the 14-3-3γ proteins strongly suggests that they play a role in mitosis. Using immunostaining, we demonstrated that 14-3-3γ filaments and clusters were associated with filamentous actin molecules. The association of 14-3-3γ proteins with F-actin is supported by other studies. For example, when adrenal chromaffin cells were incubated with purified brain 14-3-3 proteins, 14-3-3γ was detected in triton-insoluble cytoskeletal fractions by Western blot [24]. In addition, chromaffin cells incubated with purified brain 14-3-3 proteins caused F-actin to be reorganized into intracellular foci [12]. These data indicate that 14-3-3 proteins might be associated with the reorganization of F-actin. In dividing astrocytes, F-actin was concentrated inside the 14-3-3 clusters. Thus, we speculate that the cluster of 14-3-3γ proteins might serve as centers for F-actin organization. As F-actin plays a critical role during mitosis, 14-3-3γ proteins might therefore regulate mitosis by associating with F-actin.

It is proposed that 14-3-3 is capable of modifying target protein localization to bridge different proteins together [2,3]. In *C. elegans*, the pattern of 14-3-3 distribution determined the distribution of other signaling proteins and played a critical role in development [25]. In astrocytes, we found that 14-3-3γ was able to bind with phosphorylated Raf (submitted for publication) and Bad (data unpblished). Thus, we believe that the distinct distribution and the organization of 14-3-3γ proteins in astrocytes during mitosis and ischemia might also affect other signaling pathways such as those mediated by Raf or Bad. Using co-IP, we demonstrated that there was an increased amount of 14-3-3γ proteins bound to soluble actin in astrocytes under prolonged ischemic incubation. This suggested that binding between 14-3-3γ and actin played an important physiological role under stressful insults.

14-3-3 proteins were considered as anti-apoptotic because they bind to Bad, FKHRL1, and RSK and prevent apoptosis induced by these apoptotic proteins [2,3]. Overexpression of dominant-negative 14-3-3 genes [26] or peptides [27] that inhibit interactions between 14-3-3 and other proteins induced apoptosis in fibroblast and Cos7 cells, respectively. In astrocytes, overexpression of sense or antisense 14-3-3γ constructs prevented or promoted apoptosis under ischemia (data unpublished). Thus, 14-3-3γ proteins played a protective role in ischemic astrocytes. After 2 h of ischemic incubation, association between 14-3-3γ proteins and filamentous actin was evident and no astrocytes underwent apoptosis at this stage. After 6 h of ischemic incubation, apoptosis was prominent [22]. In the apoptotic cells, 14-3-3γ proteins aggregated and their concentration decreased in the cytoplasm. Previous studies demonstrated that artificial inhibition of 14-3-3 expression caused disruption [13] and clustering [12] of F-actin. This occurred in apoptotic astrocytes during ischemia. It has been known that the disorganization of F-actin was a critical step in apoptosis [16–18] and the association of 14-3-3 proteins with certain proteins such as cdc25 [28] could prevent their degradation under stress. We propose that the association of 14-3-3γ proteins with soluble and F-actin might protect actin and this would prevent apoptosis of astrocytes under ischemia.

Acknowledgments

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