

14-3-3 γ Is Upregulated by In Vitro Ischemia and Binds to Protein Kinase Raf in Primary Cultures of Astrocytes

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KEY WORDS RNA arbitrarily primed PCR; differential gene expression; injury; LY294002; U0126

ABSTRACT The 14-3-3 protein family comprises critical regulatory molecules involved in signaling during cell division, proliferation, and apoptosis. Despite extensive study, the functions of the 14-3-3 proteins in brain remain unclear. 14-3-3 γ , a subtype of the 14-3-3 family of proteins, was thought to be brain- and neuron-specific. Using RNA arbitrarily primed PCR, we identified an upregulated cDNA fragment of the 14-3-3 γ gene in primary cultures of astrocytes. Using Northern blot analysis, we confirmed this fragment was brain-specific. In cultures of astrocytes, 14-3-3 γ genes and proteins were differentially expressed at different ages and the proteins were distributed only in the cytoplasm. These results indicated that 14-3-3 γ was not neuron-specific but also expressed in astrocytes. The function of this protein in brain is unclear. Northern and Western blot analyses demonstrated that 14-3-3 γ mRNA and protein were upregulated in cultured astrocytes in an anaerobic chamber-induced ischemia model. The induction of 14-3-3 γ proteins was neither suppressed by an MAP kinase inhibitor (U0126) nor a PI-3 kinase inhibitor (LY294002). These data indicated that induction of 14-3-3 γ might not involve PI-3 and MAP kinase-dependent pathways. Using coimmunoprecipitation, we demonstrated that endogenous 14-3-3 γ bound to c-Raf-1 and p-Raf 259. As Raf is one of the critical serine/threonine kinases controlling cell growth, differentiation, and death, the binding of 14-3-3 γ to Raf indicates the critical role of this protein in ischemia-induced apoptosis and the changes in signal transduction in astrocytes in culture. *GLIA* 42:315–324, 2003. © 2003 Wiley-Liss, Inc.

INTRODUCTION

14-3-3, a group of acidic proteins about 29–33 kDa, was originally identified as brain-specific proteins in 1967 (Moore and Perez, 1967). Although they comprise about 1% of soluble brain protein (Boston et al., 1982), the functional role of 14-3-3 in brain remains elusive. Recently, the study of 14-3-3 has attracted great interest as these proteins can bind to and regulate the functions of serine/threonine-phosphorylated proteins (Freed et al., 1994; Zha et al., 1996; Brunet et al., 1999;

Light et al., 2002; Savitsky and Finkel, 2002). Accumulating evidence supports the idea that 14-3-3 are critical signaling proteins in cell division, cell cycle regula-

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Received 4 June 2002; Accepted 7 October 2002

DOI 10.1002/glia.10185

tion, and apoptosis (Baldin, 2000; Fu et al., 2000; van Hemert et al., 2001). We have also shown that 14-3-3 γ is associated with actin during cell division (Chen and Yu, 2002).

In mammalian cells, seven subtypes of 14-3-3 (β , ϵ , γ , η , σ , τ , and ζ) have been identified. Although β , γ , and η are expressed in brain (Moore and Perez, 1967; Boston et al., 1982; Isobe et al., 1991; Watanabe et al., 1993), only the γ isoform is highly brain-specific (Moore and Perez, 1967; Isobe et al., 1991; Watanabe et al., 1993), whereas the β and ζ isoforms can also be found in rat spleen and thymus (Watanabe et al., 1993). The γ isoform is mainly expressed in several areas of rat brain, such as cerebral cortex, central gray matter, superior colliculi, and pontine nuclei, and was considered to be neuron-specific (Watanabe et al., 1993). To our knowledge, little information about the expression, regulation, and functional roles of 14-3-3 γ has been reported.

Ischemia causes both neuron and astrocyte death (Wyllie et al., 1984; Benchoua et al., 2001; Northington et al., 2001; Suzuki et al., 2001). We have shown previously that astrocytes could die from apoptosis in an *in vitro* ischemia model (Yu et al., 2001). We have also shown that *in vitro* ischemia affected the expression of cytokines (Yu and Lau, 2000; Lau and Yu, 2001), heat shock proteins (Yu et al., 1995), and the activation of Akt and Erk (Jiang et al., 2002) in cultured astrocytes. In this study, we identified the presence of 14-3-3 γ , a regulatory protein in various signaling pathways, in primary culture of astrocytes and demonstrated that the expression level of both its gene and protein was upregulated under *in vitro* ischemia. The binding of 14-3-3 γ to phosphorylated Raf, an important protein kinase involved in cell survival and death (Freed et al., 1994; Irie et al., 1994; Light et al., 2002), suggests a critical role of 14-3-3 γ in astrocytes during ischemia.

MATERIALS AND METHODS

Primary Cultures of Cerebral Cortical Astrocytes

The cultures were prepared from the cerebral cortex of newborn ICR mice (Animal Care Center, Hong Kong University of Science and Technology) as reported previously (Yu et al., 2001; Jiang et al., 2002). For each 100 mm Falcon tissue culture dish (Becton Dickinson), 10 ml of cell suspension was plated. All cultures were incubated in a Napco CO₂ incubator (Precision Scientific) at 37°C with 95% air/5% CO₂ (v/v) and a humidity of 95%. 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) FCS afterward. Cultures were not used for experiments until they were at least 4 weeks old. This type of culture has been used extensively in studies of gene expression and other functions of astrocytes under injury conditions (Yu et al., 2001; Wu and Yu, 2000; Yu

and Lau, 2000; Ho et al., 2001; Lau and Yu, 2001; Chen and Yu, 2002; Jiang et al., 2002).

Anaerobic Chamber-Induced Ischemia and Treatment With Inhibitors

The use of an anaerobic chamber to induce ischemia in cultures has been reported in various studies (Yu and Lau, 2000; Ho et al., 2001; Yu et al., 2001; Chen and Yu, 2002; Jiang et al., 2002). Briefly, ischemia media (free of glucose and serum) were degassed for 30 min with N₂ and regassed with N₂:CO₂:H₂ (85:5:10) for 20 min before use. The ischemia media and cultures were then transferred into an anaerobic chamber (Forma Scientific) saturated with N₂:CO₂:H₂ (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 (HI9142, Hanna Instruments, Italy) and 0.1% (v/v) as detected by an MSA gas meter (Passport Personal Alarm), 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. This ischemia model is different from other known *in vitro* severe hypoxia models by having cultures incubated in a reduced volume of medium, thus minimizing the dilution of toxic byproducts by the large extracellular volume of medium used in other *in vitro* models. Therefore, this model is designed to simulate closely physiological ischemic conditions (Yu et al., 1995). All culture dishes were wrapped with parafilm to prevent evaporation during incubation. As the expression of 14-3-3 γ proteins was not significantly altered in cultures incubated in ischemia media for 6 h under normal incubation conditions, we used cultures without exposure to *in vitro* ischemia (0 h) as controls for the Northern and Western blotting analysis.

Inhibitors LY294002 (Cell Signaling Tech) at a final concentration of 20 μ M or U0126 (Cell Signaling Tech) at a final concentration of 10 μ M (Jiang et al., 2002) were added to the cultures just before ischemia incubation. Dimethylsulfoxide was added to the control.

RNA Arbitrarily Primed PCR (RAP-PCR)

To search for genes associated with ischemia, the patterns of gene expression in astrocytes under normal and ischemic conditions were compared using an RAP-PCR technique (Li et al., 2001, 2002). Total RNA from primary astrocyte cultures was isolated with Trizol reagent according to the manufacturer's protocol (Gibco-BRL). Reverse transcription was performed at 37°C for 1 h using 1 μ g of total RNA, 20 μ M of dNTP, 1 μ M of random hexamers, and 10 U/ μ l of Superscript II RNase H⁻ reverse transcriptase (Gibco-BRL) in a total volume of 10 μ l. PCR was performed in a reaction mixture of 10 μ l containing 1 \times PCR buffer, 200 μ M of dNTPs, 400 nM of primer mixtures, and 0.04 U/ μ l Taq

DNA Polymerase (Gibco-RBL) for 40 cycles: 94°C for 30 s, 40°C for 1 min, and 72°C for 30 s (5 min for the last cycle). The mixture of three primers included two arbitrary primers 5'-CTTGTACGCGTGTGCGAC-3' (B1) and 5'-ACGCACACGCACAGAGAG-3' (B4) obtained from an RAP-PCR Kit (Stratagene), and a primer 5'-GTCGTCGAATTCCAYACHHHRGAGAA-GCC-3' (Zn4) based on a conserved zinc finger domain. After separation on a 6% denaturing polyacrylamide gel, PCR products were visualized by ethidium bromide staining. Differentially expressed bands were excised and eluted from the gel. One of the bands, named 3.1, was reamplified by PCR, cloned, and sequenced as described below.

Cloning and Sequencing

Reamplified cDNA of band 3.1 was cloned into a pTag vector (R&D System, U.K.) and sequenced using an ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer) with either the M13 forward or reverse sequencing primer. This cDNA sequence was compared with other sequences available in the GenBank DNA databases and was verified to be identical to the 3' untranslated region of mouse 14-3-3 γ cDNA, suggesting the presence of 14-3-3 γ transcripts and proteins in astrocytes. The presence of 14-3-3 γ transcripts in astrocytes was proved when we successfully amplified the coding region of 14-3-3 γ genes from the total RNA extracted from ischemic astrocytes using the high-fidelity Taq_ELONG_{ASE} Enzyme (Gibco-BRL). Reverse transcription was performed as described above followed by 25 cycles of PCR with the *Eco*RI-flanked forward primer, 5'-GATGGATC-CATGGTGGACCGCGAGCAACTA-3' (GenBank accession number AF058799; mouse 14-3-3 γ cDNA position 183–223), and the *Bam*HI-flanked reverse primer, 5'-GCTGAATTCTTAGTTGTTGCC TTCACCGCC-3' (mouse 14-3-3 γ cDNA position 906–926). This 14-3-3 γ cDNA was digested with *Eco*RI and *Bam*HI and inserted into a *Bgl*III-*Eco*RI linearized plasmid pEGFP-N1 (Clontech). The plasmid was then sequenced as described above using forward primer 5'-CCCCATTGACGCAAATGGGCG-3' and reverse primer 5'-TGGGCACCAACCCCGGTGAACA-3'.

Northern Blot Analysis

For RNA blotting analysis, whole brains were taken from ICR mice at embryonic day 16 (E16) and neonatal and adult stages (Animal Care Center, Hong Kong University of Science and Technology). Peripheral tissues were also obtained from neonatal and adult mice. Total RNA was isolated from tissues and primary cultures of astrocytes using Trizol reagent according to the manufacturer's instructions (Gibco-BRL). Ten micrograms of total RNA was electrophoresed on a dena-

turing 1% (w/v) agarose/6.6% (v/v) formaldehyde gel and transferred onto a Hybond-N⁺ membrane (Amersham, U.K.). Blotted membranes were prehybridized at 42°C for 4 h and then probed with ³²P-labeled cDNA of clone 3.1. The probe was labeled with [α -³²P]dCTP (NEN Life Science Products) by a random hexamer procedure using the Oligolabelling Kit (Pharmacia Biotech). Hybridization was carried out at 42°C for another 14 h in a hybridization solution with a specific activity of 1×10^6 cpm/ml (Liquid Scintillation Counter Reckbeta 1209, Pharmacia). After hybridization, membranes were washed twice with $1 \times$ sodium chloride/sodium citrate (SSC) and 0.1% (w/v) SDS at room temperature (RT) for 15 min each and then washed twice with $0.1 \times$ SSC and 0.1% (w/v) SDS at 65°C for 30 min each. Autoradiography was performed by exposing the membrane to Fuji SuperRX film between two intensifying screens at -80°C overnight.

Western Blot Analysis

Cell lysate was prepared by lysing cells in ice-cold lysis buffer consisting of 150 mM NaCl, 0.1% (w/v) Triton, 20 mM Tris (pH 7.6), 0.1 mM phenylmethylsulphonyl fluoride, 0.7 $\mu\text{g/ml}$ leupeptin, and 0.5 $\mu\text{g/ml}$ pepstatin. Ten micrograms of total protein were boiled and resolved on 12% SDS-PAGE minigels under reducing conditions. They were then electrophoretically transferred onto nitrocellulose membranes. The membranes were blocked with 5% (w/v) nonfat dried milk in TBST buffer [0.1 M Tris-HCl, pH 8.0, 0.9% (w/v) NaCl, 0.1% (v/v) Tween-20] and were probed with diluted rabbit polyclonal antibodies to 14-3-3 γ , c-Raf-1 (Santa Cruz), p-Raf 259 (Cell Signaling Tech), in TBST for 2 h at RT. After incubation with goat antirabbit HRP (1/4,000; Amersham, France) for 1 h at RT, antigens were revealed by chemiluminescence reaction (Amersham). Blots were routinely stripped in a denaturing buffer (Tris-HCl 0.5 M, pH 6.8, SDS 10%, β -mercaptoethanol) and reprobbed with antibodies to actin (Santa Cruz) as a loading control. Quantitative results were expressed as a ratio of 14-3-3 γ to actin concentration.

Coimmunoprecipitation

Four hundred micrograms of total soluble proteins (cell lysate) from the astrocyte culture were used in coimmunoprecipitation. Lysates were first precleared by 10 μl of protein A agarose for 3 h at 4°C with minor agitation. After centrifugation at 3,000 rpm, supernatants were incubated with 4 μg of 14-3-3 γ or Raf antibodies at 4°C overnight. Twenty microliters of protein A agarose were added and incubated for another 3 h as previously described. After centrifugation, supernatants were discarded and the precipitates were washed three times with lysis buffer. After final centrifugation, 20 μl of protein loading buffer was added to the precipitates and boiled. Supernatants were subjected to

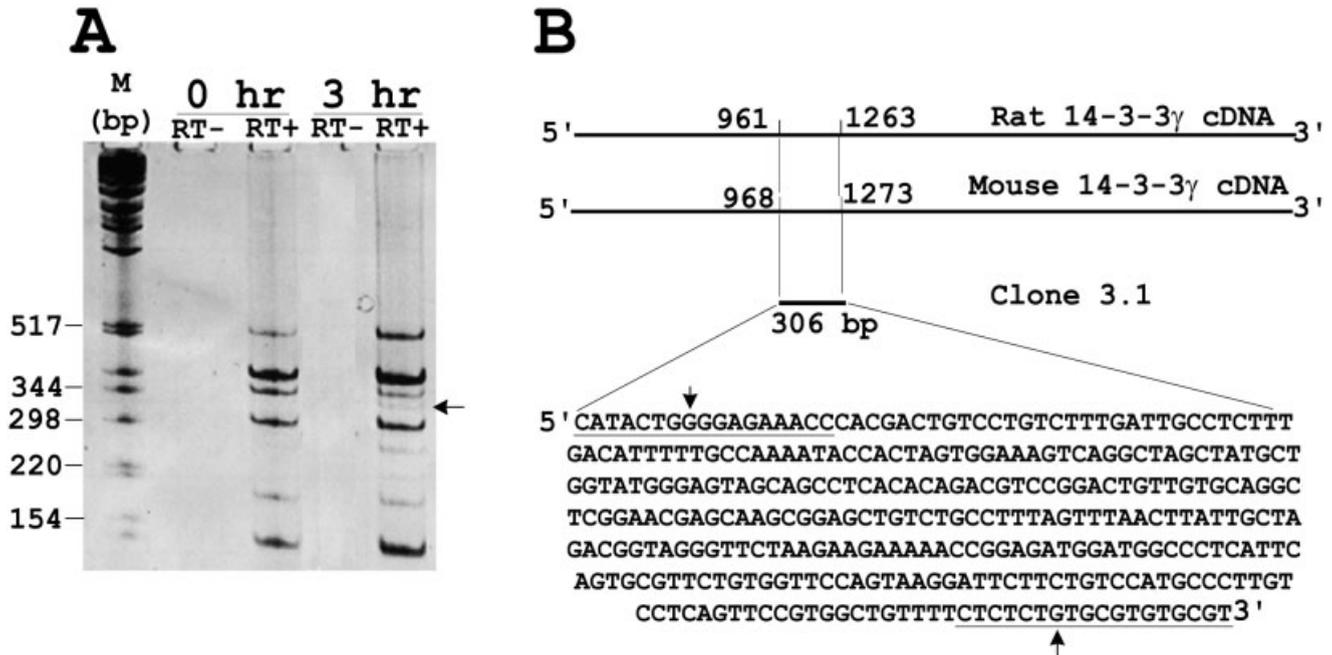


Fig. 1. Identification of 14-3-3 γ in astrocytes. **A:** Identification of 14-3-3 γ gene from RAP-PCR results. RAP-PCR was performed with total RNA extracted from astrocytes at 0 h and 3 h of ischemia with (RT⁺) or without (RT⁻) reverse transcriptase. RT⁻ reactions were used as controls to show possible sample contamination by genomic DNA. Several differentially expressed bands were identified in a single run. Only bands of suitable size were excised and eluted from the gel, reamplified with the same primer combination as for RAP-PCR, and cloned into pTag vectors for sequencing. Arrow indicates the differentially expressed band 3.1 (later identified as 14-3-3 γ)

about 320 bp in length. **B:** The nucleotide sequence of clone 3.1. Alignments of clone 3.1 with the mouse and rat 14-3-3 γ are shown. Clone 3.1 is 306 nucleotides in length. Primer Zn4 and B4 (underlined) are complementary to the 5' and 3' end of clone 3.1, respectively. Clone 3.1 was identical to a fragment of mouse 14-3-3 γ cDNA within its 3' untranslated region (UTR) and was 91% similar to the rat 14-3-3 γ cDNA. The first and the last nucleotides of the cDNA sequences compared are indicated by arrows (clone 3.1) or labeled (rat and mouse 14-3-3 γ cDNA), respectively.

Western blot analysis with various antibodies as described above. Antibodies to glial-derived growth factor (GDNF) were used as the negative control.

Immunostaining

Immunostaining was performed at 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% BSA for 2 h. After incubation with 14-3-3 γ antibodies for 2 h, the cells were washed three times with PBS and incubated with FITC-conjugated secondary antibodies for 1 h. After extensive washing, Hoechst 33342 (2 μ g/ml) was used to stain the nucleus for 5 min before observation.

Statistical Analysis

All data were presented as means \pm SEM of at least three independent experiments from different batches of cultures. Statistical analysis was performed with student's *t*-test. Unpaired *t*-test and a 95% confidence

interval were used. *P* values \leq 0.05 were considered statistically significant.

RESULTS

Identification and Cloning of Mouse 14-3-3 γ in Ischemic Cortical Astrocytes

In an RAP-PCR experiment, several differentially expressed bands were identified in astrocytes under ischemia (Fig. 1A). One band (clone 3.1) that contained 325 nucleotides (Fig. 1B) was identified and its sequence was compared with those available in GenBank. A region 309 bp long (nucleotides 8–316) in clone 3.1 was identical to the 3' untranslated region (position 961–1263) of a 3,410 bp mouse 14-3-3 γ cDNA (GenBank accession number BC008129). Clone 3.1 also had 91% nucleotide sequence similarity with the 3' untranslated region (nucleotides 961–1263) of rat 14-3-3 γ mRNA (GenBank accession number S55305). Screening with a rat brain cDNA library, and subsequent analyses with Southern blot, in addition to sequencing, indicated that only the γ and not other 14-3-3 isoforms could hybridize to clone 3.1. These results suggested that clone 3.1 could be used as a specific probe to detect the γ isoform of 14-3-3 in subsequent Northern analysis experiments.

Using total RNA extracted from ischemic astrocytes as templates, we successfully amplified the coding region of 14-3-3 γ . Ten individual clones were sequenced and the predicted amino acid sequence of these clones was also identical to that of rat and mouse 14-3-3 γ proteins. As the registered sequences of mouse 14-3-3 γ genes were obtained from a cDNA library derived from brain, it is unclear whether they were the same in neurons and astrocytes. Our data indicate that the brain-specific isoform of 14-3-3 γ genes was also expressed in astrocytes.

Characterization of 14-3-3 γ in Mouse Tissues and Primary Cultures of Astrocyte

Tissue distribution of 14-3-3 γ transcripts in mouse tissues has not been studied previously. We studied the expression of 14-3-3 γ in mouse brains at E16 and neonatal and adult stages, and in various tissues from neonatal and adult mice by Northern blot analysis. Using clone 3.1 as a probe, a major band about 3.4 kb in length was detected in brains from E16, neonatal, and adult mice (Fig. 2A). The size of the band indicated that it was a 14-3-3 γ transcript. The level of 14-3-3 γ mRNA in the brain of adult mice was found to be slightly lower than that in the brain of newborn mice. The expression of 14-3-3 γ was detected neither in the muscle, heart, kidney, stomach, liver, and lung of adult mice, nor in the heart, kidney, liver, and lung of the newborn mice. These results confirmed the brain specificity of 14-3-3 γ genes.

14-3-3 γ proteins were characterized in astrocytes with a specific 14-3-3 γ antibody. Western blot analysis showed a single band about 33 kDa in cultured astrocytes at all ages, corresponding to the size of 14-3-3 γ protein as deduced from its cDNA (Fig. 2B). 14-3-3 γ proteins were already detected in astrocytes at day 7 when the cell density in the culture dishes was still low. From days 7 to 14, astrocytes proliferated rapidly and reached confluence, and the level of 14-3-3 γ proteins increased markedly as seen at day 14. After day 14, astrocytes did not proliferate further, and the protein levels declined at days 21 and 28. The cellular distribution of endogenous 14-3-3 γ proteins was studied by immunostaining, which indicated that these proteins were mainly distributed in the cytoplasm (Fig. 2C).

Upregulation of 14-3-3 γ in Primary Cultures of Astrocytes Under Ischemia

The level of 14-3-3 γ transcripts and proteins in primary cultures of astrocyte after various periods of ischemic incubation was measured and quantified (Fig. 3). Using the cDNA of clone 3.1 as a probe, a low level of 14-3-3 γ mRNA was detected in astrocytes without ischemic incubation (0 h). The level of 14-3-3 γ mRNA doubled after 1 h of incubation and increased to more than

threefold after 2 and 4 h of incubation (Fig. 3A). The expression of 14-3-3 γ proteins in astrocytes under an extended period (8 h) of ischemia was analyzed by Western blot analysis (Fig. 3B). A low level of 14-3-3 γ proteins was detected in astrocytes before incubation under ischemic conditions. Consistent with the changes in the level of 14-3-3 γ transcripts, there was a prominent increase of 14-3-3 γ proteins in ischemic astrocytes during the first 4 h of incubation. After 2 h of ischemic incubation, the degree of injury increased and the level of 14-3-3 γ proteins also increased more than threefold. After 4 h of incubation, 14-3-3 γ protein reached its highest level, then declined after 6 and 8 h of incubation, although its level was still higher than that of culture that did not experience ischemic incubation (0 h).

Expression of 14-3-3 γ Proteins in the Presence of LY294002 and U0126

Previously, we demonstrated that Akt and Erk were activated in astrocytes under ischemia and the use of LY294002 and U0126 altered the expression of Bcl-2 in the same in vitro ischemic culture system (Jiang et al., 2002). To investigate whether these two signaling pathways are involved in the upregulation of 14-3-3 γ , we studied the effects of PI-3 and MAP kinase-specific inhibitors LY294002 and U0126 on the expression of 14-3-3 γ proteins (Fig. 4). The PI-3 and MAP kinase pathways were activated as the levels of both p-Akt and p-Erk increased after 4 h of ischemic incubation in the vehicle control, consistent with our previous study (Jiang et al., 2002). A corresponding increase in the level of 14-3-3 γ proteins also occurred, consistent with previous results of Western blotting analysis (Fig. 3B). In the presence of LY294002 and U0126, PI-3 and MAP kinase activities were suppressed markedly as compared with the vehicle control. However, levels of 14-3-3 γ proteins were not suppressed significantly in the presence of these inhibitors. These data suggest that the synthesis of 14-3-3 γ proteins in astrocytes might not be PI-3 and MAP kinase pathway-dependent.

Physiological Binding of 14-3-3 γ to c-Raf and p-Raf 259 in Astrocytes

Raf is a critical protein kinase in Ras-Raf-MAPK pathway and its function is regulated by binding to 14-3-3 (Freed et al., 1994; Irie et al., 1994; Light et al., 2002). It is important to determine whether the binding of endogenous 14-3-3 γ and Raf proteins occurs in cells in order to further our understanding of the function of 14-3-3 γ . Coimmunoprecipitation of astrocyte lysates using 14-3-3 γ antibodies and subsequent detection with antibodies specific to c-Raf-1 and p-Raf 259 indicated that both proteins could bind to 14-3-3 γ in astrocytes (Fig. 5A). In reciprocal coimmunoprecipitation using antibodies specific to c-Raf-1, binding of 14-

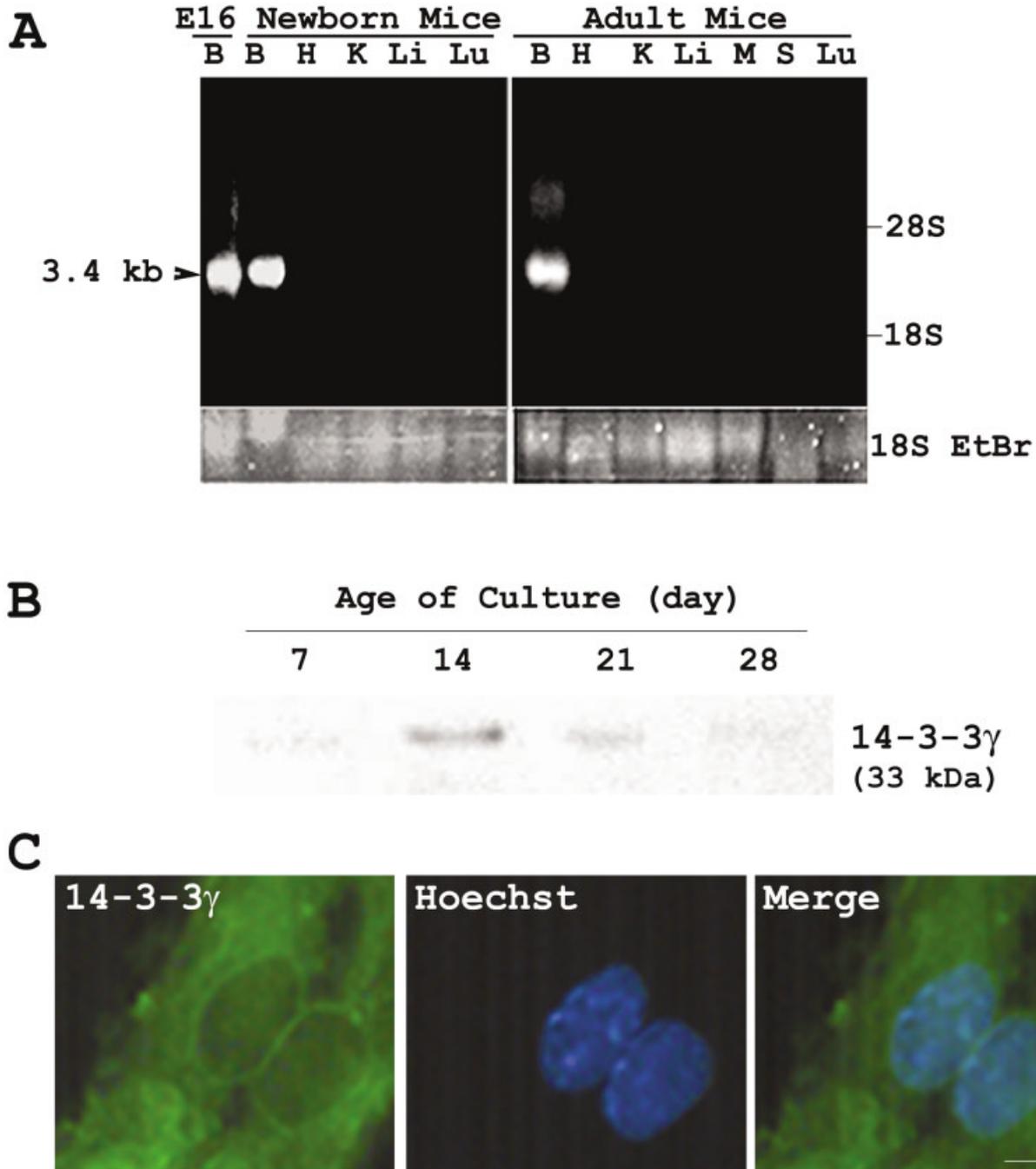


Fig. 2. Expression of 14-3-3 γ in mouse tissues and astrocytes. **A:** Characterization of 14-3-3 γ transcripts in mouse tissues and brains at various developmental ages. Total RNA was extracted from various tissues from embryonic (E16), neonatal, and adult mice. Equal amounts of total RNA were used for Northern blot analysis with clone 3.1 as the probe. 18S EtBr was used as an indicator of equal RNA loadings. 28S and 18S indicate the position of 28S and 18S ribosomal subunits, respectively. Arrow indicates a 3.4 kb band corresponding to the size of 14-3-3 γ transcripts expressed only in brain. B, brain; H, heart; K, kidney; Li, liver; Lu, lung; M, muscle; S, stomach. **B:** Characterization of 14-3-3 γ proteins in astrocytes at various developmental ages by Western blot analysis. Total protein was extracted from

primary cultures of astrocytes at day 7, 14, 21, and 28. Equal amounts of total protein were loaded on each lane, subjected to electrophoresis on a 12% PAGE gel, and transferred to a nitrocellulose membrane. The membrane was probed with a specific 14-3-3 γ antibody. A single band about 33 kDa was detected. **C:** Characterization of 14-3-3 γ protein in cortical astrocytes by immunostaining. Astrocytes were immunostained with specific 14-3-3 γ antibody and Hoechst simultaneously. Phase contrast micrographs of the same field are 14-3-3 γ staining, Hoechst staining, and their merged image. 14-3-3 γ protein mainly located in the cytoplasm of astrocytes. Scale bar, 20 μ m. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com].

3-3 γ with c-Raf was also verified (Fig. 5B) when probed with 14-3-3 γ antibodies. The use of GDNF antibody as the negative control also confirmed the binding specificity between Raf and 14-3-3 γ proteins.

DISCUSSION

14-3-3 γ was previously considered to be brain- and neuron-specific in several species (Moore and Perez, 1967; Isobe et al., 1991; Watanabe et al., 1993). We confirmed the brain specificity of 14-3-3 γ in mice. In

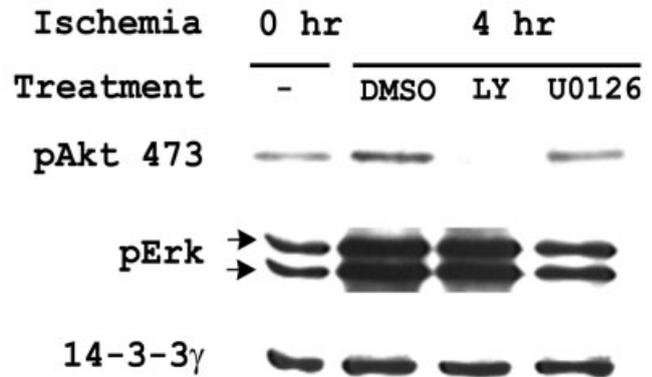
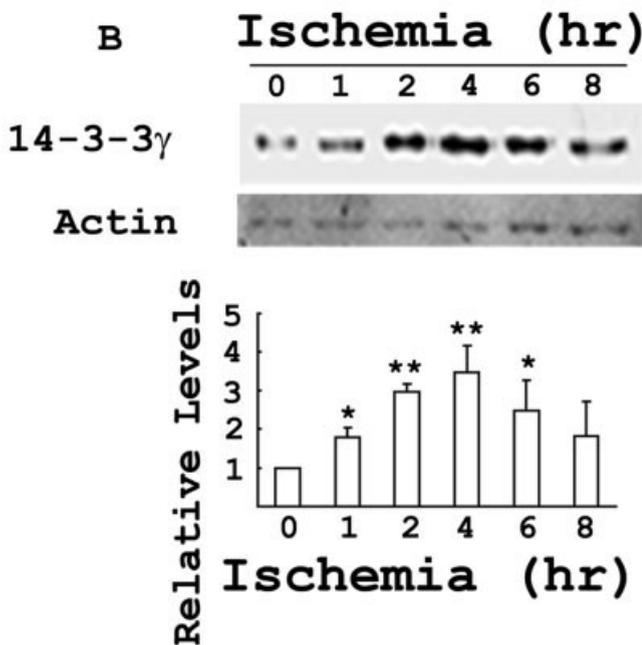
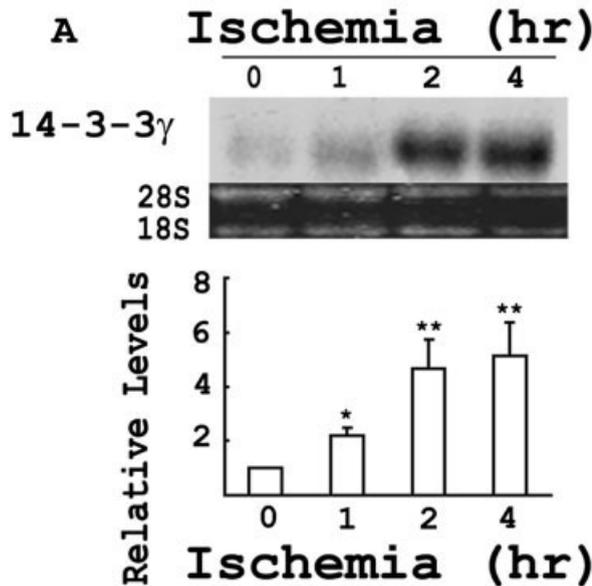


Fig. 4. Expression of 14-3-3 γ proteins in the presence of LY294002 and U0126. LY294002 (LY) and U0126 at a final concentration of 20 and 10 μ M, respectively, were added to the culture just before ischemic incubation. An equal volume of DMSO was used as an inhibitor vehicle control. Western blot analysis was performed and probed with 14-3-3 γ antibodies. The expression of 14-3-3 γ proteins did not change in the presence of either inhibitor.



this study, we identified a differential expression of 14-3-3 γ genes in astrocytes grown in primary culture of high purity under in vitro ischemia. The 14-3-3 γ gene cloned from its transcript was identical to those cloned from mouse brain. Northern and Western blotting analysis detected the expression of 14-3-3 γ transcripts and proteins in cultured astrocytes, respectively. Immunocytochemical staining showed that 14-3-3 γ proteins localized mainly in the cytosol of cultured astrocytes (Fig. 2). These results suggest that 14-3-3 γ was not neuron-specific.

Ischemia-induced gene expression is believed to be critical in determining apoptotic death in ischemic brain (Lipton, 1999; Read et al., 2001). Read et al. (2001) summarized data from about 60 differentially expressed genes identified in ischemic brain. Of these, only 18 were found to be altered at both transcriptional

Fig. 3. Induction of 14-3-3 γ proteins in astrocytes under ischemia. **A:** Increase of 14-3-3 γ transcripts in ischemic astrocytes. Equal amounts of total RNA extracted from cultures at 0, 1, 2, and 4 h of ischemia were used for Northern blotting analysis. 28S and 18S rRNA were used as indicators of equal RNA loading. Using clone 3.1 as a probe, a single band about 3.4 kb was detected. Expression of the 14-3-3 γ transcripts increased after 1 h of ischemia. The intensity of bands in the autoradiogram was measured by densitometric scanning and the level of 14-3-3 γ transcripts in ischemic astrocytes was compared with that of control astrocytes (0 h). Statistical analysis of relative expression levels showed a significant increase in 14-3-3 γ transcripts after 1, 2, and 4 h of ischemia. Data represent the mean \pm SE of results from three independent experiments. Asterisk, $P < 0.05$; double asterisk, $P < 0.01$ vs. controls. **B:** Increase of 14-3-3 γ proteins in ischemic astrocytes. Equal amounts of total protein (10 μ g) from astrocytes at 0, 1, 2, 4, 6, and 8 h of ischemia were subjected to Western blot analysis. 14-3-3 γ proteins increased after 1 h of ischemia. The same membrane after stripping was probed with antibodies to β -actin for internal control. The intensity of bands in the autoradiogram was measured by densitometric scanning and the relative levels of 14-3-3 γ protein to actin at various time points of ischemia were compared with that of control astrocytes (0 h). Statistical analysis of their relative expression levels showed a significant increase of 14-3-3 γ proteins after 1, 2, 4, and 6 h of ischemia. Data represent the mean \pm SE of results from three independent experiments. Asterisk, $P < 0.05$; double asterisk, $P < 0.01$ vs. controls.

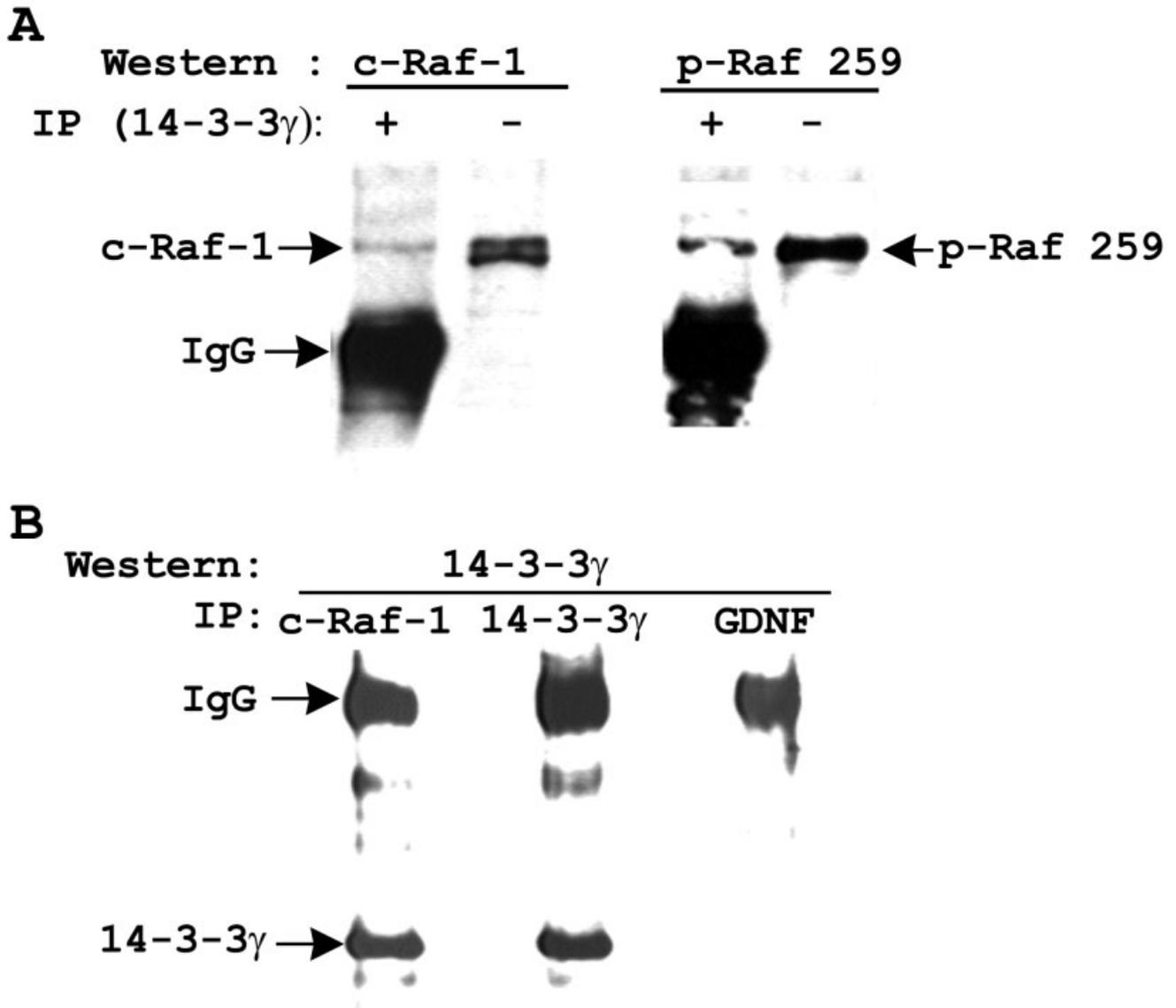


Fig. 5. Binding of endogenous 14-3-3 γ with c-Raf-1 and p-Raf 259 in reciprocal coimmunoprecipitation. Four micrograms of c-Raf-1 antibodies (A and B), 14-3-3 γ antibodies (B), or GDNF antibodies (B), were used for coimmunoprecipitation with 400 μ g of total soluble astrocyte lysate proteins. Antibodies to c-Raf-1 or p-Raf 259 were used in Western blot analysis (A) to detect Raf proteins coprecipitated with

14-3-3 γ . Twenty micrograms of cell lysates without 14-3-3 γ IP (-) treatment were used as positive controls (A). Antibodies to 14-3-3 γ were used in Western blot analysis (B) to detect 14-3-3 γ coprecipitated with c-Raf-1 (left lane). IP treatment with 14-3-3 γ and GDNF antibodies were used as positive and negative controls, respectively. No 14-3-3 γ was found to coprecipitate with GDNF (right lane).

and translational levels in ischemia. In our *in vitro* ischemic culture system, cell death did not occur before 4 h of *in vitro* ischemia and more than half of cells died at 6 h of ischemic incubation (Yu et al., 2001; Jiang et al., 2002). The temporal profile of gene expression could be followed easily in our system (Yu and Lau, 2000; Lau and Yu, 2001; Jiang et al., 2002). In this study, Northern and Western blotting analysis demonstrated that both 14-3-3 γ mRNA and protein increased about a single fold after 1 h of ischemia, i.e., much earlier than the occurrence of cell death at 4 h of ischemia (Fig. 3). The expression of 14-3-3 γ mRNA and protein in cultured astrocytes increased further to three- to fourfold

at 2 and 4 h of ischemia. These results showed clearly that 14-3-3 γ genes and proteins were inducible in astrocytes under *in vitro* ischemia. Our preliminary results (data not shown) indicated also that 14-3-3 γ proteins in cerebral cortical neuronal culture and in the cerebral cortex of newborn mice could be induced under *in vitro* and *in vivo* ischemic conditions, respectively. These results suggest that 14-3-3 γ genes and proteins might be inducible in cerebral ischemia.

Previous studies indicated that distinct isoforms of the 14-3-3 family might be induced by different kinds of injuries. The σ isoform was associated with DNA damage and genotoxic stress (Samuel et al., 2001), while

the ξ , but not the γ , isoform was altered in axotomy of nerve (Namikawa et al., 1998). In this study, we believed that 14-3-3 γ induction might be ischemic-specific. Induction of 14-3-3 γ proteins did not occur when cultured astrocytes were incubated with serum- and glucose-free media for 6 h without hypoxia or subjected to scratch wound and heat shock injuries (data not shown).

How 14-3-3 proteins are regulated in mammalian cells is unclear. PI-3/Akt (Cantley, 2002) and MAP/Erk (Irving and Bamford, 2002) are two well-known signaling pathways that regulate gene expression in various systems. Previously, we found that the activation of both pathways was associated with the expression of Bcl-2 in our *in vitro* ischemia model (Jiang et al., 2002). In this study, we found that the specific inhibitors LY294002 and U0126 for PI-3 and MAP kinases, respectively, could not block the induction of 14-3-3 γ proteins, although they effectively blocked the activation of Akt and Erk, respectively (Fig. 4). This suggests that the ischemia-induced expression of 14-3-3 γ proteins might be independent of the PI-3/Akt and MAP-Erk-pathways.

The exact functions of 14-3-3 isoforms in brain are unclear. Previous studies were largely focused on the binding properties of 14-3-3 proteins with other proteins (Irie et al., 1994; Zha et al., 1996; Brunet et al., 1999; Master and Fu, 2001; Savitsky and Finkel, 2002) in cell lines using biochemical or overexpression techniques, which, however, did not address the physiological functions of their interactions in mammalian cells. In the present study, we demonstrated that endogenous 14-3-3 γ bound with c-Raf-1 and p-Raf 259 in astrocytes grown in primary cultures (Fig. 5). Raf has at least three 14-3-3-binding sites involved in the regulation of its activity (Fu et al., 2000; van Hemert et al., 2001). The binding of 14-3-3 proteins with Raf is required for Ras-mediated Raf recruitment and subsequent MEK activation (Freed et al., 1994; Irie et al., 1994). In astrocytes under *in vitro* ischemia, the MAP/Erk pathway was activated (Fig. 4). The binding of 14-3-3 γ and Raf might play a role in the activation of the MAP/Erk pathway in our system.

14-3-3 is considered to be antiapoptotic (Xing et al., 2000; Master and Fu, 2001). Blocking the interaction of 14-3-3 proteins with other proteins, e.g., Raf, by overexpression of specific inhibitory peptides in a mammalian Cos-7 cell line could induce apoptosis. Blocking the function of 14-3-3 proteins in fibroblasts by overexpression of 14-3-3 genes also induced apoptotic death (Xing et al., 2000). This effect might be associated with the MAPK pathway. In addition to mediating apoptotic signaling pathways, 14-3-3 proteins might protect other proteins such as Cdc25 from degradation following stress (Savitsky and Finkel, 2002). Our preliminary results show that overexpression of 14-3-3 γ could promote survival of cultured astrocytes under *in vitro* ischemia (data not shown). We speculate that the induction of 14-3-3 γ proteins might be associated with

the survival of cultured astrocytes under *in vitro* ischemia.

ACKNOWLEDGMENTS

The authors thank Dr. Richard Collins from Hong Kong DNA Chips Limited for assistance in preparation of this article. Supported by grants from the Hong Kong Research Grants Council (HKUST6177/97M), the National Science Foundation of China (30270426), Beijing Natural Science Foundation (703026), and the North American Medical Association Foundation, Hong Kong (NAMA 94/95.SC01) to A.C.H.Y.

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