Role of Zinc Metallothionein-3 (ZnMt3) in Epidermal Growth Factor (EGF)-induced c-Abl Protein Activation and Actin Polymerization in Cultured Astrocytes*

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Background: Zinc plays a major role in neurochemistry, and Mt3 is one of the major regulators of neuronal zinc. However, biological functions of Mt3 are not well characterized.

Results: ZnMt3 regulates EGF-induced c-Abl activation and actin polymerization in astrocytes.

Conclusion: ZnMt3 may be a key protein for cell signaling in the CNS.

Significance: This study has demonstrated a novel signaling role for ZnMt3.

Recent evidence indicates that zinc plays a major role in neurochemistry. Of the many zinc-binding proteins, metallothionein-3 (Mt3) is regarded as one of the major regulators of cellular zinc in the brain. However, biological functions of Mt3 are not yet well characterized. Recently, we found that lysosomal dysfunction in metallothionein-3 (Mt3)-null astrocytes involves down-regulation of c-Abl. In this study, we investigated the role of Mt3 in c-Abl activation and actin polymerization in cultured astrocytes following treatment with epidermal growth factor (EGF). Compared with wild-type (WT) astrocytes, Mt3-null cells exhibited a substantial reduction in the activation of c-Abl upon treatment with EGF. Consistent with previous studies, activation of c-Abl by EGF induced dissociation of c-Abl from F-actin. Mt3 added to astrocytic cell lysates bound F-actin, augmented F-actin polymerization, and promoted the dissociation of c-Abl from F-actin, suggesting a possible role for Mt3 in this process. Conversely, Mt3-deficient astrocytes showed significantly reduced dissociation of c-Abl from F-actin following EGF treatment. Experiments using various peptide fragments of Mt3 showed that a fragment containing the N-terminal TCPCP motif (peptide 1) is sufficient for this effect. Removal of zinc from Mt3 or pep1 with tetrakis(2-pyridylmethyl)ethylenediamine abrogated the effect of Mt3 on the association of c-Abl and F-actin, indicating that zinc binding is necessary for this action. These results suggest that ZnMt3 in cultured astrocytes may be a normal component of c-Abl activation in EGF receptor signaling. Hence, modulation of Mt3 levels or distribution may prove to be a useful strategy for controlling cytoskeletal mobilization following EGF stimulation in brain cells.

Actin is a cytoskeletal protein that provides vital mechanical support to cells. In addition, an increasing body of evidence supports the possibility that actin polymerization is involved in dynamic cellular changes. For instance, actin polymerization in response to a variety of stimuli has been demonstrated to play key roles in intracellular protein trafficking, cell migration, adhesion, and proliferation (1, 2). Moreover, actin polymerization is involved in cell signaling, notably through effects on the endocytosis of membrane receptors (3). To form microfilaments, monomeric globular actin (G-actin) polymerizes in an ATP-dependent manner into filamentous actin (F-actin), which has two distinct polarized ends. Two parallel F-actin strands create the double helix structure of the microfilament (1). The F-actin microfilaments then undergo further dynamic changes involving elongation, depolymerization, nucleation and severing, capping, or branching of termini, all of which contribute to various morphological changes of cells, including membrane ruffling and retraction, and the formation of membrane protrusions, microspikes, microvilli, lamellipodia, and stress fibers (2, 4). Numerous and diverse proteins modulate actin organization. For example, thymosin binds to G-actin to prevent it from polymerizing, whereas profilin promotes monomeric addition to the barbed plus-end of the F-actin filament (5–8). Thus, the sum of the effects of all actin-binding proteins determines actin dynamics at any given moment.

As is the case for several other growth factor receptors, activation of the epidermal growth factor receptor causes changes in actin polymerization. A large number of proteins are involved in EGF-induced actin reorganization, one of which is c-Abl. c-Abl, a member of the nonreceptor tyrosine kinase family (9), was originally characterized as a proto-oncogene (10) and as such has been implicated in several clinicopathological disorders, including chronic myeloid leukemia (11). c-Abl possesses Src homology 2 and 3 and F-actin-binding domains (12). Therefore, c-Abl may control actin dynamics either by phosphorylating cytoskeletal proteins or by direct binding to F-actin (13, 14). The first evidence linking c-Abl to actin organization was the presence of significant morphological defects in the actin latticework of abl/arg<sup>−/−</sup> mouse embryos (15). c-Abl has
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self-inhibitory properties, controlling its own activity through conformational changes (9). F-actin binds the inactive form of c-Abl, which is released upon c-Abl activation and actin polymerization. Hence, F-actin polymerization and c-Abl may reciprocally regulate each other (1).

Recent evidence indicates that zinc plays a major role in neurochemistry. Of many zinc-binding proteins, metallothioneins (Mts) are regarded as the major regulators of cellular zinc. Of four isoforms (Mt1–4), metallothionein-3 (Mt3) is most prominently expressed in the central nervous system (16, 17); it is also expressed in cultured astrocytes (18–22). Mt3 binds many different proteins, including actin (23, 24). However, the biological function of Mt3, especially regarding the actin-Mt3 binding, has not been characterized. Here, we assessed the role of Mt3 as an actin-binding protein in EGF-stimulated astrocytes, specifically testing the hypothesis that Mt3 is involved in c-Abl-induced cytoskeletal alterations.

EXPERIMENTAL PROCEDURES

Chemicals and Antibodies—EGF was purchased from R&D Systems (Minneapolis, MN). Cytochalasin D, N,N,N′,N′-tetraakis(2-pyridylmethyl)ethylenediamine (TPEN),2 zinc chloride, and α-tubulin antibody were obtained from Sigma (St. Louis, MO), STI-571 was obtained from LC Laboratories (Woburn, MA). Antibodies against phosphorylated CrkL (p-CrkL) and c-Abl (p-c-Abl) were obtained from Cell Signaling Technology (Danvers, MA). Anti-c-Abl antibodies were obtained from BD Biosciences (Franklin Lakes, NJ) and Merck (Whitehouse Station, NJ). The antibodies against GFP and CrkL were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and Novus Biologicals (Littleton, CO). Actin Biochem kits for assaying F-actin polymerization and c-Abl may reciprocally regulate each other (1).

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Zn-TPEN Complex—The whole procedures were the same as in the previous report (25) with a minor modification. In brief, TPEN was premixed with an equimolar quantity of ZnCl2 to form the 1:1 Zn-TPEN complex.

Animals—WT and Mt3-null mice were produced by mating male heterozygous C57B16/129sv hybrid mice (–/+) with female heterozygous C57B16/129sv hybrid mice (–/+). The offspring of matings were genotyped by PCR using the following primers.

1. Forward primer (5′-GATACACCAGCAGGTCTCAGGGTCCATG-3′)
2. Reverse primer (5′-CATGGACCCTGAGACCTGCTGGTG-3′)

The resulting PCR products were digested with EcoRI and PstI. The final product of mutated plasmid was confirmed by DpnI digestion. The final product of mutated plasmid was confirmed by sequencing and Western blotting.

Cortical Tissue Preparation—Brain tissues were obtained from 3-month-old WT (+/+ and Mt3-null (–/–) mice. For cortical brain collection, cortex was removed from whole brain tissue, frozen immediately on dry ice, and stored at −80 °C.

Western Blotting Analysis—Protein expression levels were measured in whole-cell extracts of cultured cortical astrocytes and cortical brain tissues. Cortical brain tissue and astrocytes obtained from both WT and Mt3-null mice, cultured for 14–21 days in vitro, were sonicated in lysis buffer (20 mm Tris-Cl (pH 7.4), 150 mm NaCl, 1 mm EDTA, 1 mm EGTA, 1% (v/v) Triton X-100, 2.5 mm sodium pyrophosphate, 1 mm Na3VO4, 1 µg/ml leupeptin, and 1 mM PMSF). After centrifugation, protein concentrations in supernatants were determined by the bicinchoninic acid (BCA) method (Thermo Scientific, Rockford, IL). For Western blotting, equal amounts of protein were separated by SDS-PAGE on polyacrylamide gels and transferred to polyvinylidine difluoride membranes. Immunoreactive proteins were visualized using an enhanced chemiluminescence kit (Pierce) (Rockford, IL), and the intensity of protein bands was analyzed densitometrically to quantitatively assess protein expression. All experiments with cultured astrocytes were repeated at least three times using cultures from neonatal mice born from different females.

Construction of Mutant Mt3 Plasmid—A mutated version of Mt3 (GFP-mMt3) was created by Cosmo Genetech (Seoul, Republic of Korea) using the modified Stratagene QuikChange mutagenesis protocol (Invitrogen). To change the N-terminal amino acid sequences from “TCPCP” to “TCWCI,” the mutagenic primers 5′-CATGGACCCTGAGACCTGCTGGTG-TATCACTGTGTGTTCC-3′ and 5′-GGAACACCATG-GATACACGACAGGTCAGCTCATG-3′ were used. Following PCR, the amplification product was digested with DpnI. The final product of mutated plasmid was confirmed by sequencing and Western blotting.

Silingence of Metallothionein Genes—Stealth RNAi target sequences for mouse Mt1, Mt2, and Mt3 were chemically synthesized by Invitrogen. Astrocytes were transiently transfected with Stealth RNAi against Mt1, Mt2, and Mt3 using Lipofectamine RNAiMAX (Invitrogen), as suggested by the manufacturer. Forty eight hours after transfection, the specific silencing of individual Mts in astrocytes was confirmed by real-time-PCR.

Real Time PCR—Total RNA was extracted from astrocytes with TRIzol® reagent (Invitrogen). QuantiTect® reverse transcription kit (Qiagen, Hilden, Germany) was used to generate cDNA according to the manufacturer’s instructions. Samples were run as quadruplicates for 40 cycles using 7300 Real Time PCR systems (Applied Biosystems). PCR was performed using the SYBR® Premix Ex Taq™ master mix (Takara Bio, Japan) and forward and reverse primers for Mt1, Mt2, and Mt3. Ther-
mocycle conditions included initial denaturation at 95 °C for 5 s, followed by 40 cycles at 55 °C for 10 s and 72 °C for 31 s. Values were normalized to that of the housekeeping gene β2-microglobulin.

**Immunocytochemistry and Confocal Microscopy**—Astrocytes were fixed with 4% (v/v) paraformaldehyde for 20 min and permeabilized by incubating with 0.2% (v/v) Triton X-100 containing 1% (w/v) bovine serum albumen (BSA) in phosphate-buffered saline (PBS) for 10 min. After washing with PBS, astrocytes were blocked with PBS containing 1% BSA. For co-staining of F-actin, c-Abl, and β-catenin, astrocytes were incubated overnight with primary antibodies (for c-Abl and β-catenin) and then subsequently incubated with secondary antibodies conjugated to Alexa Fluor 488 for c-Abl or 555 for β-catenin (Invitrogen) and 200 nm Alexa Fluor 633-phalloidin (Invitrogen), a selective dye for F-actin, for 1 h at room temperature. After counterstaining, cells were incubated with Hoechst 33342 dye (Invitrogen) for 5 min. After incubation, cells were washed three times with PBS and mounted with a mounting media (Dako, Glostrup, Denmark). Stained cells were observed with a confocal fluorescence microscope (Leica TCS-SP2, Wetzlar, Germany) equipped with a laser-imaging system using four different filters.

**Transfection of Plasmid DNAs**—Cortical astrocytes were used at 14–21 days in vitro. Cultured astrocytes were transiently transfected with GFP-C1, as a control construct, or GFP-Mt3, which generated from rat brain cDNAs, or GFP-mMt3, using Lipofectamine 2000 (Invitrogen) as suggested by the manufacturer. Twenty four hours after transfection, the cells were collected and lysed for experiments.

**Purification of c-Abl and GFP-Mt3**—c-Abl and GFP-Mt3 from primary mouse astrocyte cultures were prepared according to the following methods. In brief, 24 h after transfecting, vehicle-transfected (control) astrocytes or astrocytes transfected with GFP-Mt3 were solubilized by incubating in HNEG buffer (50 mM HEPES (pH 7.5), 150 mM NaCl, 1 mM EDTA, 10% glycerol, 1% Triton X-100, 10 μg/ml aprotinin, 10 μg/ml pepstatin A, and 1 mM phenylmethylsulfonyl fluoride (PMSF)) for 30 min on ice. After centrifugation of cell lysates to pellet debris, the supernatant was collected, and its protein concentration was determined by the BCA method mentioned above.

Whole-cell lysates were then incubated with c-Abl antibody or GFP antibody together with protein A/G-agarose beads for 48 h at 4 °C. The resulting immune complex was washed three times with washing buffer (20 mM HEPES (pH 7.5), 150 mM NaCl, 1 mM EDTA, 10% glycerol, 0.1% Triton X-100, 10 μg/ml aprotinin, 10 μg/ml pepstatin A, and 1 mM PMSF). c-Abl and GFP-Mt3 were eluted from the beads with elution buffer (10 mM lysine (pH 11), 150 mM NaCl, 1 mM EDTA, 10% glycerol, and 0.1% Triton X-100). The eluates were immediately neutralized by adding 0.1 volume of 1 M HEPES (pH 7.4).

**Actin Co-sedimentation Assay**—Actin sedimentation assays were performed following the manufacturer’s instructions (Cytoskeleton, Inc.) with minor modifications. Briefly, 250 μg of nonmuscle actin was mixed with 250 μl of general actin buffer (G-buffer) containing 5 mM Tris-HCl (pH 8.0) and 0.2 mM CaCl2 and incubated on ice for 30 min. For preparation of F-actin stock, 25 μl of actin polymerization buffer containing 100 mM Tris-HCl (pH 7.5), 500 mM KCl, 20 mM MgCl2, and 10 mM ATP was added to the nonmuscle actin mixture and incubated for an additional 1 h at room temperature. After incubation, purified protein was centrifuged at 150,000 × g for 1 h at 4 °C, and supernatants were collected. Purified proteins were combined with F-actin stock and incubated for an additional 30 min at room temperature, after which all tubes were centrifuged at 150,000 × g for 1.5 h at 22 °C. The supernatants were carefully removed and added to 5× Laemmli reducing sample buffer. Pellets were resuspended in 1× Laemmli reducing sample buffer and boiled for 5 min at 95 °C. The extent of actin binding and actin polymerization activity were determined by Western blotting of proteins obtained from pellets and supernatants.

**Actin Polymerization Activity Assay**—Actin polymerization assays were also performed following manufacturer’s instructions (Cytoskeleton, Inc.) with minor modifications. In brief, 200 μg of purified protein was centrifuged at 150,000 × g for 1 h at 4 °C. The resulting supernatant was removed and stored on ice until used for experiments. For G-actin stock preparation, 1125 μl of ice-cold G-buffer containing ATP was mixed with a 5-μl frozen aliquot of pyrene actin and incubated on ice for 1 h to depolymerize actin oligomers that had formed during storage. After centrifuging actin at 14,000 rpm at 4 °C for 30 min, the supernatant was removed and transferred to a new tube. G-actin stock (100 μl) was combined with individual protein mixtures and applied to 96-well plates. After shaking samples for 5 s, fluorescence was read at excitation and emission wavelengths of 350 and 407 nm, respectively, once every 60 s for a total of 20 min to establish base-line fluorescence values. Thereafter, 2 μl of actin polymerization buffer (100 mM Tris-HCl (pH 7.5), 500 mM KCl, 20 mM MgCl2, and 10 mM ATP) was added to individual wells. Actin polymerization activity was determined by plotting fluorometer readings. Every experiment was repeated at least three times and averaged for statistical analyses.

**Statistics**—All data were presented as means ± S.E. For multiple comparisons among groups, one-way analysis of variance followed by Fisher LSD post hoc test was employed. Paired t tests were used to analyze differences between two groups. p values < 0.05 were considered statistically significant.

**RESULTS**

Levels of Activated c-Abl and Those of Its Substrate CrkL Are Altered in Cultured Astrocytes and Cortical Tissues of Mt3-null Mice—Basal levels of activated (phosphorylated) c-Abl were slightly elevated in cultured Mt3-null astrocytes, but interestingly, treatment with EGF failed to increase p-c-Abl levels and only slightly increased the levels of p-CrkL, a substrate of c-Abl, in these cells (Fig. 1A). In contrast, EGF treatment of WT astrocytes significantly increased the levels of both p-c-Abl and p-CrkL (Fig. 1A). A similar pattern of changes was obtained using mouse cortical tissues. Cortical tissues from Mt3-null mice exhibited significantly reduced levels of p-c-Abl and p-CrkL (Fig. 1B). These findings indicate that Mt3 may be involved in EGF-induced/c-Abl-mediated signaling in the brain, which is critical for cell proliferation, migration, and cytoskeletal regulation.
Abl Kinase and F-actin Are Interdependently Regulated in WT Astrocytes—Actin polymerization is one of the central events in actin filament organization, which in turn mediates cellular processes such as protein endocytosis and the subsequent intracellular protein trafficking and recycling of various cellular membrane proteins (1, 2). Interestingly, it has been demonstrated that F-actin and c-Abl, which is an F-actin-binding protein (26–28), are reciprocally regulated (1). To examine this, we used STI-571, an inhibitor of Abl kinase. Addition of STI-571 eliminated EGF-induced F-actin polymerization (Fig. 2A). In addition, EGF-induced dissociation of c-Abl from F-actin was completely blocked by co-treatment with STI-571 (Fig. 2B). In addition, to test whether F-actin polymerization is essential for EGF-induced c-Abl activation, we treated WT astrocytes with cytochalasin D (CytD), a potent inhibitor of

FIGURE 1. Reduced activation of c-Abl and its substrate (CrkL) in Mt3-null astrocytes. A, Western blotting for phospho-c-Abl (p-c-Abl) and p-CrkL in cortical astrocytes cultured from WT (+/+ ) and Mt3-null (−/− ) mice after treating with 100 ng/ml EGF for the indicated times. A Western blot for α-tubulin was presented as a loading control. Bars denote changes in the density ratio of p-c-Abl or p-CrkL relative to that of nonphosphorylated c-Abl or CrkL. All ratio values were normalized to the ratio in EGF-ununtreated WT controls, defined as 1 (*, p < 0.05; **, p < 0.01 versus WT 0 min or KO 0 min; n = 4 cultures). B, Western blotting for p-c-Abl and p-CrkL in cortical brain tissues obtained from 3-month-old WT (+/+ ) and Mt3-null (−/− ) mice. Bars indicate changes in the density ratio of p-c-Abl or p-CrkL relative to that of each non-phosphorylated protein (*, p < 0.05; **, p < 0.01; n = 5 and n = 4 cortices for WT and Mt3-null mice, respectively).

FIGURE 2. Abl kinase and F-actin interaction. A, confocal fluorescence micrographs of WT astrocytes stained with Alexa Fluor 633 phalloidin (F-actin) and Hoechst 33342 (nucleus). Cells from WT and Mt3-null mice were treated for 15 min with vehicle only, 10 μM STI-571 alone, 100 ng/ml EGF, or EGF plus STI-571. EGF-induced actin polymerization was completely inhibited by STI-571. Scale bar, 20 μm. B, Western blotting for c-Abl after actin co-sedimentation assays. Cultured astrocytes from WT mice were treated for 15 min with vehicle or 100 ng/ml EGF alone or 10 μM STI-571 plus EGF. Lysates were incubated with F-actin and centrifuged to separate proteins bound to F-actin aggregates. The resulting pellets were immunoblotted with an anti-c-Abl antibody. The values shown represent fold changes in the density ratio of c-Abl band relative to that in controls (CTL), defined as 1 (**, p < 0.01 versus CTL; n = 5 cultures). C, c-Abl activation by EGF requires actin polymerization. WT astrocytes were preincubated without or with 1 μM CytD, an inhibitor of actin polymerization, for 1 h. After pretreatment with CytD, cells were incubated with 100 ng/ml EGF alone or EGF plus CytD for the indicated times. Bars denote relative changes in the density ratio for p-c-Abl relative to that of c-Abl (*, p < 0.05 versus CytD 0 min control; **, p < 0.01 versus no-CytD 0 min control; n = 5 cultures).
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actin polymerization that acts by capping the F-actin tail. Interestingly, as was the case with Mt3-null astrocytes (Fig. 1A), the basal level of p-c-Abl was somewhat elevated in CytD-treated cells compared with controls. Surprisingly, EGF treatment substantially reduced the level of p-c-Abl to below basal levels (Fig. 2C), demonstrating that F-actin polymerization is required for EGF-induced c-Abl activation.

**Interdependent Regulation of Abl Kinase and F-actin Is Altered in Mt3-null Astrocytes**—Prior to addressing the possibility that F-actin and c-Abl interactions are altered in Mt3-null astrocytes, we first examined these interactions in WT astrocytes. As reported by others (29), c-Abl was partly co-localized with F-actin near the plasma membrane (PM) of WT astrocytes under basal conditions; this co-localization was substantially reduced following treatment with EGF (Fig. 3B). Western blotting for c-Abl in actin co-sedimentation assays confirmed that the amount of c-Abl able to bind to F-actin was decreased in WT cells following EGF treatment (Fig. 3A). A densitometric analysis of Western blots showed that EGF treatment reduced the ratio of c-Abl in the pellet, consistent with the finding that, upon activation, the amount of c-Abl dissociated from polymerized F-actin increased (1).

Next, we examined whether the reduction in EGF-induced c-Abl activation in Mt3-null astrocytes involved a change in the binding of c-Abl and F-actin. Actin co-sedimentation assays revealed that, unlike WT astrocytes, where the association between c-Abl and F-actin was diminished upon EGF treatment, Mt3-null cells instead exhibited a slight increase in the association between F-actin and c-Abl (Fig. 3A). This lack of dissociation between F-actin and c-Abl following EGF treatment was also observed in astrocytes doubly stained with phalloidin (for F-actin) and an antibody to c-Abl (Fig. 3B). F-actin and c-Abl co-localization was noted in the vicinity of the PM in both WT and Mt3-null astrocytes prior to EGF treatment, indicating that some c-Abl may be bound to peri-PM F-actin. Upon EGF treatment, WT astrocytes exhibited a degree of F-actin polymerization in association with the disappearance of peri-PM co-localization. In contrast, little F-actin polymerization occurred in Mt3-null astrocytes, and the peri-PM co-localization of F-actin and c-Abl remained less changed (Fig. 3B).

**Mt3 Binds to Polymerized F-actin Following EGF Treatment**—Because the association of c-Abl and F-actin following EGF treatment was altered in Mt3-null astrocytes (Fig. 3), we examined whether Mt3 binding to actin (23, 30) was responsible for this change. To accomplish this, we transfected WT astrocytes with GFP-Mt3 or GFP-C1 plasmid DNA (negative control) and incubated cells with phalloidin to stain for F-actin. Whereas GFP-C1 fluorescence was completely unchanged by EGF treatment, GFP-Mt3 fluorescence shifted from a diffuse pattern to a more centralized pattern upon EGF treatment (Fig. 4A). Polymerized F-actin showed similar concurrent changes, resulting in a substantial overlap between the two signals (Fig. 4A). To further confirm this, we conducted actin co-sedimentation assays. In GFP-Mt3-overexpressing astrocytes, GFP-Mt3 protein was present in the pellet fraction after pelleting polymerized F-actin, indicating that Mt3 was associated with polymerized F-actin. This interaction was enhanced after stimulation with EGF (Fig. 4C), whereas it was not altered by GFP-C1 transfection. These results support the idea that Mt3 can associate with polymerized F-actin, suggesting that this interaction may be involved in the changes in c-Abl/F-actin interaction in Mt3-null astrocytes noted above.

**Mt3 Enhances F-actin Polymerization and Dissociation of c-Abl from F-actin**—Next, we examined whether Mt3 affects the F-actin polymerization processes per se. For this, we performed in vitro actin polymerization assays, which are useful for characterizing the role of putative actin-binding proteins in the actin polymerization process. Interestingly, different from GFP-C1 (Fig. 5A) that had no effect, GFP-Mt3 purified from GFP-Mt3-transfected cultured astrocytes and added to the assay mixture increased actin polymerization in an Mt3 concentration-dependent manner (Fig. 5B), suggesting that binding of Mt3 to F-actin promotes actin polymerization.

We then examined whether Mt3 affects the association of c-Abl and F-actin by transfecting WT astrocytes with GFP-Mt3 or GFP-C1 plasmid DNA and then examining the binding of c-Abl and F-actin. Overexpression of GFP-Mt3 negatively
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**FIGURE 4.** Mt3 associates with polymerized F-actin in EGF-stimulated WT astrocytes. A, confocal fluorescence micrographs of GFP-C1 or GFP-Mt3 co-stained with Alexa Fluor 633 phalloidin and Hoechst 33342 (blue) in WT astrocytes. Before staining, cells were exposed to vehicle or 100 ng/ml EGF for 15 min (n = 5 cultures). Scale bar, 20 μm. B and C, Western blotting for GFP in pellets prepared from actin co-sedimentation assays of lysates from GFP-C1- (B) or GFP-Mt3-overexpressing astrocytes (C). Samples were exposed to vehicle or 100 ng/ml EGF for 15 min (not significant (NS) versus no-EGF GFP-C1; n = 5 cultures, **, p < 0.01 versus no-EGF GFP-Mt3; n = 5 cultures).

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**FIGURE 5.** Mt3 enhances F-actin polymerization. A and B, bars denote the degree of actin polymerization. Actin polymerization was induced by incubating different concentrations (0–10 μg) of purified GFP-C1 (A) or GFP-Mt3 (B) with a G-actin stock solution containing actin polymerization buffer (APB). Ratios of fluorescence intensities obtained at excitation and emission wavelengths of 350 and 407 nm, respectively, were normalized to the ratio in the GFP-C1- (A) or GFP-Mt3-deficient group (B), defined as 1 (not significant (NS) versus no-GFP-C1; n = 4 cultures, *, p < 0.05; **, p < 0.01 versus no-GFP-Mt3; n = 4 cultures).

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modulated the interaction between c-Abl and F-actin (Fig. 6A). Direct application of purified GFP-Mt3, not GFP-C1, to purified c-Abl also attenuated the interaction between c-Abl and F-actin (Fig. 6, B and C). Although further studies are required to establish whether the Mt3 effect is attributable to the induced actin polymerization or some other mechanism, such as direct displacement of c-Abl, Mt3 clearly promotes the dissociation of c-Abl from F-actin.

Specific Peptide Sequence of Mt3 Is Responsible for Regulating the Interaction between c-Abl and Polymerized F-actin—To determine which portion of the Mt3 protein is responsible for the observed effects on F-actin and c-Abl, we synthesized four different peptide fragments of the full-length Mt3 sequence (Fig. 7A). Interestingly, only the first N-terminal peptide sequence containing a TCPCP motif induced responses similar to that of Mt3 (Fig. 7B). Thus, it is likely that the N-terminal portion of the Mt3 molecule is largely responsible for the binding to F-actin and the dissociation of c-Abl from F-actin.

Mt3-induced Actin Polymerization and Dissociation of c-Abl from F-actin Require the Presence of Zinc—Both Mt3 and peptide 1 have “zinc-binding motifs” (31–34). Hence, we examined whether the binding of zinc was required for the effects of Mt3 and/or peptide 1. TPEN, a potent zinc chelator, blocked Mt3- and peptide 1-induced dissociation of c-Abl from F-actin (Fig. 8, A and B). In addition, whereas TPEN caused no reduction in F-actin polymerization in the absence of Mt3, Mt3-induced increases in actin polymerization were completely blocked by TPEN (Fig. 8C).

Mt3 can bind to copper, zinc, and cadmium (35). In terms of normal physiology, however, only copper and zinc are likely relevant. Hence, in a practical sense, we need to rule out the possibility of copper acting as a responsible metal for the current Mt3 effects. TPEN is a lipophilic cell membrane-permeant metal chelator, binding Cu^{2+} (K_D = 10^{-20} M), Zn^{2+} (K_D = 10^{-16} M), and Fe^{2+} (K_D = 10^{-15} M) with high affinity (36, 37). Because TPEN binds Cu^{2+} with higher affinity than Zn^{2+}, we also used Zn-TPEN as a control to examine whether actin mobilization in astrocytes is dependent on not Cu^{2+} but Zn^{2+} residing in Mt3. Because of the difference in binding affinity, Zn-TPEN cannot remove Zn^{2+} but can still remove Cu^{2+} from Mt3. Different from TPEN, Zn-TPEN did not significantly alter the Mt3-triggered actin polymerization as well as interaction of c-Abl to F-actin (Fig. 8, A and C). These results suggest that zinc binding is essential for the effects of Mt3 and peptide 1 on actin polymerization.

Silencing and Mutation of Mt3, but Not Mt1/2, Mimic Mt3-null Responses in Mt3 WT Astrocytes—Although our results thus far indicated that Mt3-null astrocytes exhibit reduced c-Abl activation and F-actin polymerization upon EGF treat-
ment, it is possible that these effects are the result of some uncharacterized developmental change caused by the knock-out of Mt3. To examine this possibility, we knocked down Mt3 in WT astrocytes using small interfering RNAs (siRNAs). As controls, we also knocked down Mt1 and -2 with their respective siRNAs. Because WT astrocytes had normal levels of Mt3 prior to siRNA treatment, this experiment would enable us to determine whether indirect developmental effects of Mt3 gene knock-out were important for the above-noted changes in Mt3-null cells. Whereas acute knockdown of Mt1/2 did not alter F-actin polymerization, F-actin/c-Abl binding, and c-Abl activation upon EGF treatment, siRNA-mediated Mt3 knockdown produced a pattern of changes similar to that of genetic deletion of Mt3; EGF treatment caused an increase in the amount of c-Abl able to bind to F-actin (Fig. 9D) and decreased F-actin polymerization (Fig. 9F) and c-Abl activation (Fig. 9A). To demonstrate whether mutation of Mt3, besides silencing of this, also alters the normal Mt3 effect on actin mobilization and c-Abl activation, we created a mutant GFP-Mt3 construct by substituting TCPCP in the N-terminal amino acid sequences to TCWCI. Overexpression of double point mutated Mt3 plasmid in WT astrocytes mimicked Mt3-null state in actin mobilization and association to c-Abl (Fig. 9, A, E, and F). These results support the interpretation that the changes observed in Mt3-null astrocytes were not caused by some uncharacterized developmental changes, but instead are specifically due to the loss of normal Mt3 gene function.

DISCUSSION

The central finding of this study is that Mt3, a CNS-enriched metallothionein isoform, plays an important role in c-Abl activation downstream of the EGF receptor in astrocytes. Genetic ablation or siRNA-mediated knockdown of Mt3 in cultured astrocytes resulted in a significant reduction of c-Abl activation in WT astrocytes mimicking Mt3-null state in actin mobilization and association to c-Abl (Fig. 9, A, E, and F). These results support the interpretation that the changes observed in Mt3-null astrocytes were not caused by some uncharacterized developmental changes, but instead are specifically due to the loss of normal Mt3 gene function.

FIGURE 6. Mt3 facilitates dissociation of c-Abl from polymerized F-actin. A, Western blotting for c-Abl. Actin co-sedimentation assays were performed on astrocytes from WT mice transfected with vehicle, GFP-C1, or GFP-Mt3 plasmid DNA. Separated pellets were analyzed by Western blotting for c-Abl (*, p < 0.05 versus Mock; n = 5 cultures). B and C, Western blotting for c-Abl. Purified c-Abl from astrocytes was incubated with F-actin stock alone or F-actin plus purified GFP-C1 (B) or GFP-Mt3 (C). The level of c-Abl bound to polymerized F-actin was detected by an anti-c-Abl antibody (not significant (NS) versus no-GFP-C1; n = 5, *, p < 0.05 versus no-GFP-Mt3; n = 5).

FIGURE 7. N-terminal sequence of Mt3 may regulate the interaction between c-Abl and polymerized F-actin. A, mouse Mt3 amino acid sequence: peptides 1–4 with the indicated sequences were synthesized for experiments. B, Western blotting for c-Abl. c-Abl was purified from WT astrocytes, and equal amounts were incubated with F-actin stock solution alone, F-actin plus purified GFP-Mt3, or the indicated peptides (pep1−4). After actin co-sedimentation assays, the level of F-actin-bound c-Abl was visualized and quantified with an anti-c-Abl antibody (n = 3). Of the peptides tested, only peptide 1 was as effective as full-length Mt3 in reducing the level of bound c-Abl (**, p < 0.01 versus vehicle (veh); n = 3).
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A Western blotting for c-Abl. Total cell lysates were mixed with either 1 μM TPEN or 1 μM Zn-TPEN alone or purified Mt3 alone or purified Mt3 plus TPEN or Zn-TPEN. Mixtures were incubated with F-actin stock solution and subsequently ultracentrifuged. Pellets were immunoblotted with an anti-c-Abl antibody (*, p < 0.05; **, p < 0.01 versus no-Mt3 and no-TPEN/Zn-TPEN, or versus Mt3 alone, n = 6). B Western blotting for c-Abl. Purified c-Abl solutions were mixed with 1 μM TPEN alone or with synthesized peptide 1, with or without TPEN. Mixtures were incubated with F-actin stock solution and subsequently ultracentrifuged. Pellets were immunoblotted with an anti-c-Abl antibody (*, p < 0.05; **, p < 0.01 versus no-pep1 and no-TPEN, or versus pep1 alone, n = 3). C bars denote the degree of actin polymerization. A G-actin stock containing APB was incubated without (control) or with purified GFP-Mt3 alone, GFP-Mt3 denoted the degree of actin polymerization. A G-actin stock containing APB was incubated without (control) or with purified GFP-Mt3 alone, GFP-Mt3 denoted the degree of actin polymerization. A G-actin stock containing APB was incubated without (control) or with purified Mt3 alone, or purified Mt3 plus TPEN or Zn-TPEN. Mixtures were incubated with F-actin stock solution and subsequently ultracentrifuged. Pellets were immunoblotted with an anti-c-Abl antibody (*, p < 0.05; **, p < 0.01 versus no-Mt3 and no-TPEN/Zn-TPEN, or versus Mt3 alone, n = 6). Western blotting for c-Abl. Purified c-Abl solutions were mixed with 1 μM TPEN alone or with synthesized peptide 1, with or without TPEN. Mixtures were incubated with F-actin stock solution and subsequently ultracentrifuged. Pellets were immunoblotted with an anti-c-Abl antibody (*, p < 0.05; **, p < 0.01 versus no-pep1 and no-TPEN, or versus pep1 alone, n = 3). C bars denote the degree of actin polymerization. A G-actin stock containing APB was incubated without (control) or with purified GFP-Mt3 alone, GFP-Mt3 denoted the degree of actin polymerization. A G-actin stock containing APB was incubated without (control) or with purified Mt3 alone, or purified Mt3 plus TPEN or Zn-TPEN. Mixtures were incubated with F-actin stock solution and subsequently ultracentrifuged. Pellets were immunoblotted with an anti-c-Abl antibody (*, p < 0.05; **, p < 0.01 versus no-Mt3 and no-TPEN/Zn-TPEN, or versus Mt3 alone, n = 6). Western blotting for c-Abl. Purified c-Abl solutions were mixed with 1 μM TPEN alone or with synthesized peptide 1, with or without TPEN. Mixtures were incubated with F-actin stock solution and subsequently ultracentrifuged. Pellets were immunoblotted with an anti-c-Abl antibody (*, p < 0.05; **, p < 0.01 versus no-pep1 and no-TPEN, or versus pep1 alone, n = 3). C bars denote the degree of actin polymerization. A G-actin stock containing APB was incubated without (control) or with purified GFP-Mt3 alone, GFP-Mt3 denoted the degree of actin polymerization. A G-actin stock containing APB was incubated without (control) or with purified Mt3 alone, or purified Mt3 plus TPEN or Zn-TPEN. Mixtures were incubated with F-actin stock solution and subsequently ultracentrifuged. Pellets were immunoblotted with an anti-c-Abl antibody (*, p < 0.05; **, p < 0.01 versus no-Mt3 and no-TPEN/Zn-TPEN, or versus Mt3 alone, n = 6). Western blotting for c-Abl. Purified c-Abl solutions were mixed with 1 μM TPEN alone or with synthesized peptide 1, with or without TPEN. Mixtures were incubated with F-actin stock solution and subsequently ultracentrifuged. Pellets were immunoblotted with an anti-c-Abl antibody (*, p < 0.05; **, p < 0.01 versus no-pep1 and no-TPEN, or versus pep1 alone, n = 3). C bars denote the degree of actin polymerization. A G-actin stock containing APB was incubated without (control) or with purified GFP-Mt3 alone, GFP-Mt3 denoted the degree of actin polymerization. A G-actin stock containing APB was incubated without (control) or with purified Mt3 alone, or purified Mt3 plus TPEN or Zn-TPEN. Values were normalized to the ratio in controls, defined as 1 (*, p < 0.05 versus no-purified GFP-Mt3, no-TPEN, and no-Zn-TPEN; n = 6 cultures).

FIGURE 8. Mt3- and peptide 1-induced activation of actin polymerization and dissociation of c-Abl from F-actin require zinc binding. Western blotting for c-Abl. Total cell lysates were mixed with either 1 μM TPEN or 1 μM Zn-TPEN alone or purified Mt3 alone or purified Mt3 plus TPEN or Zn-TPEN. Mixtures were incubated with F-actin stock solution and subsequently ultracentrifuged. Pellets were immunoblotted with an anti-c-Abl antibody (*, p < 0.05; **, p < 0.01 versus no-Mt3 and no-TPEN/Zn-TPEN, or versus Mt3 alone, n = 6). Western blotting for c-Abl. Purified c-Abl solutions were mixed with 1 μM TPEN alone or with synthesized peptide 1, with or without TPEN. Mixtures were incubated with F-actin stock solution and subsequently ultracentrifuged. Pellets were immunoblotted with an anti-c-Abl antibody (*, p < 0.05; **, p < 0.01 versus no-pep1 and no-TPEN, or versus pep1 alone, n = 3). C bars denote the degree of actin polymerization. A G-actin stock containing APB was incubated without (control) or with purified GFP-Mt3 alone, GFP-Mt3 denoted the degree of actin polymerization. A G-actin stock containing APB was incubated without (control) or with purified Mt3 alone, or purified Mt3 plus TPEN or Zn-TPEN. Mixtures were incubated with F-actin stock solution and subsequently ultracentrifuged. Pellets were immunoblotted with an anti-c-Abl antibody (*, p < 0.05; **, p < 0.01 versus no-Mt3 and no-TPEN/Zn-TPEN, or versus Mt3 alone, n = 6). Western blotting for c-Abl. Purified c-Abl solutions were mixed with 1 μM TPEN alone or with synthesized peptide 1, with or without TPEN. Mixtures were incubated with F-actin stock solution and subsequently ultracentrifuged. Pellets were immunoblotted with an anti-c-Abl antibody (*, p < 0.05; **, p < 0.01 versus no-pep1 and no-TPEN, or versus pep1 alone, n = 3). C bars denote the degree of actin polymerization. A G-actin stock containing APB was incubated without (control) or with purified GFP-Mt3 alone, GFP-Mt3 denoted the degree of actin polymerization. A G-actin stock containing APB was incubated without (control) or with purified Mt3 alone, or purified Mt3 plus TPEN or Zn-TPEN. Values were normalized to the ratio in controls, defined as 1 (*, p < 0.05 versus no-purified GFP-Mt3, no-TPEN, and no-Zn-TPEN; n = 6 cultures).

the CNS. This effect is unique to Mt3, because down-regulation of Mt1/2 failed to produce similar results.

One interesting aspect of the novel action of Mt3 on c-Abl is that it is likely mediated by its interaction with actin. Actin is a cytoskeletal protein that is necessary for the structural integrity of cells. However, recent evidence indicates that actin also plays a key role in diverse cellular signaling processes by modulating the activation and endocytosis/recycling/degradation of receptors (1, 2). The entire actin organization process usually occurs very rapidly in response to even minute cellular changes. Such dynamic and precise reorganization is facilitated by formation of complexes with actin-binding proteins, of which more than 200 have been identified (38, 39). The actin-binding protein c-Abl is uniquely defined by its reciprocal regulatory relationship with F-actin (1, 4, 14). In the inactive conformation, c-Abl is folded in such a way that the catalytically active domain is shielded and the actin-binding motif is exposed, supporting F-actin binding. However, following growth factor stimulation, the actin-binding motif is unfolded to reveal the catalytic domain, resulting in the release of c-Abl from F-actin, which triggers F-actin polymerization (14).

Our results from EGF-treated cultured astrocytes are consistent with this dynamic. EGF receptor activation led to concurrent c-Abl activation and F-actin polymerization. Moreover, inhibition of c-Abl inhibited F-actin polymerization and vice versa, indicating reciprocal modulation of these two proteins. Our novel finding is that Mt3 appears to participate in this process by physically interacting with F-actin. In cell-free assays, Mt3 not only bound to polymerized F-actin, it also promoted actin polymerization. siRNA-mediated silencing of Mt1/2 genes had no effect on F-actin polymerization or c-Abl activation, indicating that this effect is specific for Mt3. Taken together, these results suggest that brain-enriched Mt3 may be a modulator of F-actin-mediated c-Abl activation in brain cells, especially in astrocytes.

The mechanism by which Mt3 inhibits the association of F-actin and c-Abl is as yet undefined. However, because Mt3 not only binds to F-actin but also promotes its polymerization, it is possible that the polymerization of actin promoted by Mt3 may itself stimulate further release of c-Abl from F-actin, making more c-Abl available for activation by the EGF receptor. In this scenario, the absence and mutation of Mt3 should result in reduced F-actin polymerization, reduced c-Abl dissociation, and reduced c-Abl activation, a sequence of events that is consistent with the present results. Alternatively, Mt3 may displace c-Abl from F-actin; not only would this promote actin polymerization, it would also render c-Abl more available for phosphorylation. Future mechanistic studies are needed to elucidate the molecular relationship among Mt3, actin, and c-Abl.

Mt3 is a small (68-amino acid) protein consisting of distinct β (N-terminal) and α (C-terminal) domains (40–44). The sequence unique to Mt3 is a TCPCP motif at positions 5–9 in the N terminus (32). The fact that of all the Mt3-derived peptides only the peptide containing the TCPCP sequence mimicked the Mt3 effect on F-actin polymerization may explain why only Mt3, and not Mt1/2, has this effect.

Formation of a tightly bound metal-thiolate cluster yields a characteristic conformational change of the TCPCP sequence that may provide a potential interface for protein/protein interactions (45–47). The apo-Mt3 form (i.e. with no bound metal) is predominantly unstructured and unable to support activity; thus, the metal binding status of Mt3 is important for its biochemical and physiological activity (33). In this context, we found that removal of zinc also rendered Mt3 and peptide 1 completely incapable of promoting actin polymerization or dis-
sociating c-Abl from F-actin, suggesting that zinc binding is essential for the Mt3 effects described here.

Mt3 has previously been linked to diverse biological phenomena, including glycolytic metabolism, protein chaperone and scaffolding functions, metal transport/buffering, neurite outgrowth inhibition, and redox signaling (23, 24, 48–50). In addition to these functions, our results show that Mt3 is involved in actin dynamics and, likely as a consequence, in c-Abl signaling. Further studies will be needed to elucidate the molecular mechanism underlying these effects of Mt3.

Collectively, the results presented here support a novel role of Mt3 in brain cells, i.e. modulation of actin cytoskeleton and c-Abl activation. A growing body of evidence indicates that diverse neurological disorders, including Alzheimer disease and brain ischemia, are accompanied by altered levels of c-Abl and actin (51–57). Hence, the insight that Mt3 plays a role in

FIGURE 9. Mt3 silencing and mutation in WT astrocytes replicates changes in Mt3-null astrocytes. A, Western blotting for phospho-c-Abl (p-c-Abl) and c-Abl in cortical astrocytes cultured from Mt3 WT (+/+) mice after treating with 100 ng/ml EGF for the indicated times. Before exposure to EGF, cultured cells were transfected with stealth RNAi negative control duplexes (NC siRNA), which have been reported to have no gene silencing activity, a combination of Mt1 and Mt2 (Mt1/2) siRNA, Mt3 siRNA, and GFP-C1 or GFP-mMt3. B, bars denote changes in the density ratio of p-c-Abl relative to that in nonphosphorylated c-Abl (*, p < 0.5; **, p < 0.01 versus no-EGF 0 min-NC si or -Mt1/2 si or -GFP-C1, respectively; n = 7 cultures for Mt1/2 and Mt3 siRNA, n = 5 cultures for GFP-C1 and GFP-mMt3). C, quantitative analysis of Mt1, Mt2, and Mt3 mRNA levels by RT-PCR in WT astrocytes treated with the respective siRNAs. Bars denote the ratio of each mRNA value to the corresponding β2-microglobulin value, normalized to the ratio in negative controls (NC) (**, p < 0.01 versus negative controls; n = 3 cultures). D, Western blotting for c-Abl in pellets prepared from actin co-sedimentation assays in WT astrocytes treated with negative control siRNA (NC si), or siRNA against Mt3 (Mt3 si), or Mt1/2 (Mt1/2 si). Samples were taken before (0 min) and 15 min after treatment with 100 ng/ml EGF. Bands indicate c-Abl expression in negative control siRNA (NC si) and Mt3- or Mt1/2-silenced astrocytes. The bars indicate fold changes in the density of c-Abl band relative to that in EGF-untreated control (*, p < 0.05 versus no-EGF negative control siRNA; n = 3 cultures). E, Western blotting for c-Abl in pellets prepared from actin co-sedimentation assays in WT astrocytes transfected with GFP-C1 or GFP-mMt3. Samples were obtained before (0 min) and 15 min after exposure to 100 ng/ml EGF. Bands represent c-Abl expression in GFP-C1 and GFP-mMt3-transfected WT astrocytes. The bars show fold changes in c-Abl expression relative to that in EGF-untreated, GFP-C1-overexpressed cells, defined as 1 (*, p < 0.05 versus no-EGF GFP-C1; n = 3 cultures). F, confocal fluorescence micrographs of astrocytes treated with negative control siRNA (NC si), or siRNA against Mt1/2 (Mt1/2 si), or Mt3 (Mt3 si) stained with Alexa Fluor 633 phalloidin and Hoechst 33342. Cultures were treated with vehicle (CTL) or 100 ng/ml of EGF for 15 min (n = 5 cultures). Scale bars, 20 μm.
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c-Abl activation and actin dynamics may suggest new therapeutic strategies for such neurological disorders.

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REFERENCES

1. Woodring, P. J., Litwack, E. D., O’Leary, D. D., Lucero, G. R., Wang, J. Y., and Hunter, T. (2002) J. Cell Biol. 156, 879–892

2. Zimag, S. H. (1996) Curr. Opin. Cell Biol. 8, 66–73

3. Sotiropoulos, A., Ginestis, D., Copeland, J., and Treisman, R. (1999) Cell 98, 159–169

4. Woodring, P. J., Hunter, T., and Wang, J. Y. (2001) J. Biol. Chem. 276, 21047–21056

5. Ditsch, A., and Wegner, A. (1994) Eur. J. Biochem. 224, 223–227

6. Hartwig, J. H., Chambers, K. A., and Stossel, T. P. (1989) J. Cell Biol. 108, 467–479

7. Schafer, D. A., Jennings, P. B., and Cooper, J. A. (1996) J. Cell Biol. 135, 169–179

8. Schiwa, M., Pryzwansky, K. B., and van Blerkom, J. (1982) Philos. Trans. R. Soc. Lond. B Biol. Sci. 299, 199–205

9. Sirvent, A., Benistant, C., and Roche, S. (2008) Biochimie 100, 617–631

10. Smith, J. M., and Mayer, B. J. (2002) Front. Biosci. 7, d31–42

11. Reddy, E. P., Smith, M. J., and Srinivasan, A. (1983) Proc. Natl. Acad. Sci. U.S.A. 80, 3623–3627

12. Van Etten, R. A. (1999) Trends Cell Biol. 9, 179–186

13. Hernández, S. E., Krishnaswami, M., Miller, A. L., and Koleske, A. J. (2004) Trends Cell Biol. 14, 36–44

14. Woodring, P. J., Hunter, T., and Wang, J. Y. (2003) J. Cell Sci. 116, 2613–2626

15. Koleske, A. J., Gifford, A. M., Scott, M. L., Nee, M., Bronson, R. T., Miczek, P. T., and Janmey, P. A. (1994) Proc. Natl. Acad. Sci. U.S.A. 91, 6518–6522

16. Koleske, A. J., Gifford, A. M., Scott, M. L., Nee, M., Bronson, R. T., Miczek, P. T., and Janmey, P. A. (1994) Proc. Natl. Acad. Sci. U.S.A. 91, 6518–6522

17. West, A. K., Hidalgo, J., Eddins, D., Levin, E. D., and Aschner, M. (2008) Neurotoxicology 29, 489–503

18. Carrasco, J., Giralt, M., Penkowa, M., Stalder, A. K., Campbell, I. L., and Hidalgo, J. (2000) Exp. Neurol. 163, 46–54

19. Hidalgo, J., Aschner, M., Zatta, P., and Vasák, M. (2001) Brain Res. Bull. 55, 133–145

20. Penkowa, M., Moos, T., Carrasco, J., Hadberg, H., Molinero, A., Bluthmann, H., and Hidalgo, J. (1999) Glia 25, 343–357

21. Uchida, Y., Ibarra, Y., and Tomonaga, M. (1989) Biochim. Biophys. Res. Commun. 150, 1263–1267

22. Yu, W. H., Lukwi, W. J., Bergeron, C., Niznik, H. B., and Fraser, P. E. (2001) Brain Res. 894, 37–45

23. El Ghazi, I., Martin, B. L., and Armitage, I. M. (2006) Exp. Biol. Med. 231, 1500–1506

24. El Ghazi, I., Martin, B. L., and Armitage, I. M. (2010) Int. J. Alzheimers Dis. 2011, 208634

25. Sheridan, R. E., Deshpande, S. S. (1995) Toxicon 33, 539–549

26. Cao, H., Sanguinetti, A. R., and Mastick, C. C. (2004) Exp. Cell Res. 294, 159–171

27. den Hartigh, J. C., van Bergen en Henegouwen, P. M., Verkleij, A. J., and Boonstra, J. (1992) J. Cell Biol. 119, 349–355

28. Plattner, R., Kadlec, L., DeMali, K. A., Kazlauskas, A., and Pendergast, A. M. (1999) Genes Dev. 13, 2400–2411

29. Van Etten, R. A., Jackson, P. K., Baltimore, D., Sanders, M. C., Matsudaira, P. T., and Janmey, P. A. (1994) J. Cell Biol. 125, 325–340

30. Lahti, D. W., Hoekem, J. D., Tokheim, A. M., Martin, B. L., and Armitage, I. M. (2005) Protein Sci. 14, 1151–1157

31. Ding, Z. C., Zheng, Q., Cai, B., Ni, F. Y., Yu, W. H., Teng, X. C., Gao, Y., Liu, F., Chen, D., Wang, Y., Wu, H. M., Sun, H. Z., Zhang, M. J., Tan, X. S., and Huang, Z. X. (2008) J. Inorg. Biochem. 102, 1965–1972

32. Ding, Z. C., Ni, F. Y., and Huang, Z. X. (2010) FEBS J. 277, 2912–2920

33. Faller, P. (2010) FEBS J. 277, 2921–2930

34. Hasler, D. W., Faller, P., and Vasák, M. (1998) Biochemistry 37, 14966–14973

35. Palumaa, P., Eriste, E., Njunkova, O., Pokras, L., Jörnvall, H., and Sillard, R. (2002) Biochemistry 41, 6158–6163

36. Shumaker, D. K., Vann, L. R., Goldberg, M. W., Allen, T. D., Wilson, K. L., and Hunter, T. (1995) J. Cell Biol. 132, 1500–1506

37. McCabe, M. J., Jr., Jiang, S. A., and Orrenius, S. (1993) Lab. Invest. 69, 101–110

38. Winder, S. I., and Ayscough, K. R. (2005) J. Cell Biol. 171, 651–654

39. Pollard, T. D., and Cooper, J. A. (1986) Annu. Rev. Biochem. 55, 987–1035

40. Williamson, M. P. (1994) Biochem. J. 297, 249–260

41. Yu, H., Chen, J. K., Feng, S., Dalgarano, D. C., Brauer, A. W., and Schreiber, S. L. (1994) Cell 76, 933–945

42. Bell, S. G., and Vann, L. R. (2007) Exp. Neurol. 204, 337–347

43. Uchida, Y., and Tomonaga, M. (1989) Brain Res. 481, 190–193

44. Alvarez, A. R., Sandoval, P. C., Leal, N. R., Castro, P. U., and Kosik, K. S. (2004) Neurobiol. Dis. 17, 326–336

45. Cancino, G. I., Toledo, E. M., Leal, N. R., Hernandez, D. E., Yévenes, L. F., Inestrosa, N. C., and Alvarez, A. R. (2008) Brain 131, 2425–2442

46. Fulga, T. A., Elson-Schwab, I., Khurana, V., Steinhiibl, M. L., Spires, T. L., Hyman, B. T., and Feany, M. B. (2007) Nat. Cell Biol. 9, 139–148

47. Ing, Z., Caltagaroni, J., and Bowser, R. (2009) J. Alzheimers Dis. 17, 409–422

48. Mendoza-Naranjo, A., Gonzalez-Billault, C., and Maccioni, R. B. (2007) J. Cell Sci. 120, 279–288

49. Minamide, L. S., Striegel, A. M., Boyle, J. A., Meberg, P. J., and Bamburg, J. R. (2000) Nat. Cell Biol. 2, 628–636

50. Netzer, W. J., Dou, F., Cai, D., Veach, D., Jean, S., Li, Y., Bornmann, W. G., Clarkson, B., Xu, H., and Greengard, P. (2003) Proc. Natl. Acad. Sci. U.S.A. 100, 12444–12449