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Journal of Cellular Biochemistry 113:3019–3028 (2012)

Journal of Cellular Biochemistry

Mps One Binder 2 Gene Upregulation in the Stellation of Astrocytes Induced by cAMP-Dependent Pathway

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ABSTRACT

Astrocytes, the major glial population in the central nervous system (CNS), play an important role in neuronal homeostasis, neurogenesis, and synaptogenesis. The cells have a stellate shape with elaborated processes in the developing CNS. Cultured astrocytes become stellate when the cells undergo differentiation in response to stimuli. Nevertheless, the molecular mechanism for astrocytic stellation is poorly understood. Here, we showed that the addition of serum induced a flat polygonal shape in cultured astrocytes with a reduced level of Mps one binder 2 (Mob2) that is involved in neurite growth by forming stable complex with a nuclear Ser/Thr kinase Dbf2-related protein kinase 1 (NDR1). Furthermore, exposure to a membrane permeable cAMP analogue, dbcAMP, not only induced astrocytic stellation, but also caused an increase in Mob2 expression. Similarly, the upregulation of Mob2 mRNA expression was induced by exposure of astrocytes to pituitary adenylyl cyclase-activating polypeptide (PACAP). Pretreatment with a cAMP/protein kinase A (PKA) inhibitor, KT-5720, significantly blocked the effect of dbcAMP and PACAP on induced upregulation of Mob2 mRNA expression in astrocytes. In addition, the process withdrawal of dbcAMP-treated astrocytes was caused by the inhibition of Mob2 expression using lentivirus-mediated Mob2 shRNA delivery system. Based on our findings, we suggest that Mob2 is involved in PKA signaling-mediated astrocytic stellation. J. Cell. Biochem. 113: 3019–3028, 2012.

KEY WORDS: ASTROCYTES; Mob2; CELL STELLATION; dbcAMP; PACAP

Monopolar spindle-one-binder (Mob) family of zinc binding proteins has been found in unicellular organisms to mammals. The Mob family is involved in controlling important cellular processes, such as mitotic exit, centrosome duplication, apoptosis, and cell proliferation in eukaryotes [Stavridi et al., 2003; Ponchon et al., 2004; Lai et al., 2005; Mrkobrada et al., 2006; Hergovich et al., 2009; Ho et al., 2010]. For instance, Mob proteins in yeasts interferes mitotic exit network to regulate mitosis [Colman-Lerner et al., 2001; Citterio et al., 2005]. *Drosophila* Mob1/Mats is known to integrate Hippo tumor-suppressing pathway to inhibit cell growth and apoptosis [Lai et al., 2005; Ho et al., 2010]. Interestingly, human homolog Mob1A (hMob1A) can restore the lethality of *Drosophila mats* mutants [Lai et al., 2005], suggesting that the biological role of Mob proteins is conserved from flies to humans. Mob proteins are also thought to regulate cell morphogenesis in alteration of the actin cytoskeleton arrangement and neurite formation via the binding and activation of nuclear NDRs [Hergovich et al., 2006; Schulte et al., 2010]. Among Mob members, Mob2 has a role in photoreceptor morphological differentiation and wing hair morphogenesis in flies [He et al., 2005; Liu et al., 2009]. The recent study has addressed that Mob2 protein mediates the neuritogenesis of a mouse neuroblastoma cell line Neuro2A through the binding to NDR2 [Lin et al., 2011].

Astrocytes, the most abundant glial cell population in the CNS, play the supporting role in the regulation of neurogenesis, blood–brain barrier integrity, and neuronal metabolic/ionic homeostasis [Castonguay et al., 2001; Fields and Stevens-Graham, 2002; Garner and Zhai, 2002]. During CNS maturation, astrocytes undergo differentiation along with the extensive morphological change, which is important for their neuronal supporting function [Mason et al., 1988; Wang et al., 1994; Ghosh et al., 2005]. This process so called as stellation is associated with the development of multiple

Grant sponsor: National Science Council of Taiwan; Grant numbers: NSC-95-2311-B-006-004, NSC-99-2811-B-006-058.

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Manuscript Received: 11 October 2011; Manuscript Accepted: 26 April 2012
Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 4 May 2012
DOI 10.1002/jcb.24180 • © 2012 Wiley Periodicals, Inc.

process bearing forms. It causes astrocytes to send their stellate processes surrounding neuronal perikarya, axons, and synaptic areas, which in turn mediate neuronal excitability and synaptic plasticity [Federoff, 1986; Mason et al., 1988].

In vitro studies have indicated that increased levels of intracellular cAMP can trigger the stellation of astrocytes, which is due to the reorganization of cytoskeleton associated with proteins, tubulin, microtubule, and actin filaments [Goldman and Abramson, 1990; Baorto et al., 1992; Ramsell et al., 1996; Won and Oh, 2000]. Pituitary adenylyl cyclase-activating polypeptide (PACAP), a member of the vasoactive intestinal peptide (VIP)/glucagon peptide family, also provokes cAMP production to induce astrocytic differentiation [Vallejo and Vallejo, 2002]. Nevertheless, the molecular mechanism for astrocytic stellation is poorly understood. In this study, to examine Mob2 gene expression in stellate astrocytes, cell permeant cAMP analog dibutyryl cAMP (dbcAMP) and PACAP were used to induce astrocytic stellation. We found that Mob2 mRNA expression was increased during the promotion of astrocytic stellation by dbcAMP and PACAP. Moreover, the upregulation of Mob2 mRNA expression in dbcAMP- or PACAP-primed astrocytes was cAMP/protein kinase A (PKA)-dependent. Inhibition of Mob2 expression reduced the cell stellation of dbcAMP and PACAP-treated astrocytes, indicating that Mob2 serves as a critical molecule for astrocytic stellation.

MATERIALS AND METHODS

MATERIALS

Culture media, antibiotics, Lipofectamine 2000, plasmid pcDNA3.1, and Texas red-conjugated phalloidin were purchased from Invitrogen (Carlsbad, CA). Cell cultureware were from BD Biosciences (San Jose, CA). Fetal bovine serum (FBS) and calf serum (CS) were the products from HyClone (Logan, UT). The plasmid pEGFP-N2 vector was from Clontech (Mountain View, CA). Poly-D-lysine (PDL), dbcAMP, phenylmethylsulphonyl fluoride (PMSF), 4′,6-diamidino-2-phenylindole (DAPI) and proteinase inhibitor cocktail were from Sigma (St. Louis, MO). KT-5720 (cell permeable specific competitive inhibitor of PKA), BAPTA-AM (intracellular Ca^{2+} chelator), and PACAP were purchased from Calbiochem (Cambridge, MA). Primers used for the examination of Mob2 and cyclophilin A (CyPA) mRNA expression were synthesized by MWG Biotech AG (Ebersberg, Germany). The antibodies used in the study were purchased from the following sources: anti-GFAP from Boehringer Mannheim Biochemical (Indianapolis, IN), anti-Mob2 from Everest Biotech (Oxfordshire, UK), horseradish peroxidase (HRP) labeled anti-goat, biotinylated anti-rabbit, and biotynylated anti-mouse, FITC-labeled avidin D were purchased from Vector Laboratories (Burlingame, CA).

CELL CULTURE

Mixed glial cells were isolated from 1- to 2-day-old Sprague–Dawley (SD) rat pup cerebral cortices as described previously [Hsiao et al., 2007]. In brief, primary cells (10^7 cells/75 cm^2) were plated onto PDL-coated 75-cm^2 flasks supplied with DMEM/F-12 medium containing 10% FBS. The medium was changed every 2–3 days. Eight days later, microglia and cell debris were removed using the shake-off method [McCarthy and de Vellis, 1980]. Through immunostaining for astrocytic cell marker (glial fibrillary acidic protein; GFAP), the remaining cells in the cultures were noticed to consist of approximately 95% primary rat astrocytes. To examine Mob2 expression in astrocytes, the cells were seeded on the appropriate tissue culture dishes, and then treated with dbcAMP or PACAP in serum-free medium.

IMMUNOFLUORESCENCE

Astrcoytes (5×10^4 cells/2-cm^2) were seeded on 12-mm coverslips placed onto a 24-well tissue culture plate. Twenty-four hours after treatment, the cells were fixed in PBS containing 4% paraformaldehyde for 15 min, and then incubated with anti-GFAP antibody (1:1,000) at 4°C overnight in PBS containing 0.1% Triton X-100. The cultures were incubated with the appropriate secondary antibodies (1:200) and FITC-avidin (1:200) for 45 min, and followed by DAPI nuclear staining. The immunostained cells were observed under a fluorescence microscope (Nikon E800) with a cooling CCD camera system.

F-ACTIN STAINING

Astrcoytes (5×10^4 cells/2-cm^2) were seeded on 12-mm coverslips placed onto a 24-well tissue culture plate. After treatment, the cells were fixed in PBS containing 4% paraformaldehyde for 15 min. The cells were permeabilized in PBS containing 0.1% Triton X-100, and then incubated with Texas red-conjugated phalloidin for 1 h at room temperature. Nuclear staining was carried out using DAPI solution. F-actin staining was observed under a Nikon E800 fluorescence microscope with a cooling CCD camera system.

QUANTIFICATION OF STELLATE ASTROCYTES AND ASTROCYTIC PROCESS LENGTH

According to the description as indicated previously [Kume et al., 2009], stellate astrocytes were defined as the cells with one or more processes that were at least twice longer than the diameter of their cell bodies. The stellate astrocytes with GFAP immunostaining were counted over approximately 150 cells per culture under a Nikon E800 fluorescence microscope equipped with a cooling CCD system. Alternatively, to measure the total process length per stellate cell, the images were randomly captured from five fields per culture with a cooling CCD system. The total process length of each stellate cell was determined using Image J program (Wayne Rasband, NIMH, Bethesda, MD; http://rsbweb.nih.gov).

QUANTITATIVE REAL-TIME POLYMERASE CHAIN REACTION (Q-PCR)

Total RNAs were isolated followed by the procedure described previously [Liu et al., 2008]. Q-PCR assay for Mob2 mRNA expression was performed by Roche LightCyClerTM using Light-Cycler FastStart DNA Master SYBR Green I kit. PCR amplification of rat Mob2 was carried out for 10 min at 95°C, followed by 50 cycles set for 10 s at 95°C, annealing for 10 s at 60°C, and extension for 20 s at 72°C. Results (Cycle at threshold, Ct) were normalized to those of the housekeeping gene CyPA as ΔCt (Ct_{Mob2}–Ct_{CyPA}). Values are

expressed as the relative mRNA levels by comparison of $\Delta Ct_{treatment}$ to $\Delta Ct_{control}$ ($2^{\Delta Ct_{treatment}-\Delta Ct_{control}}$). The specific sequences for rat Mob2 are as follows: 5′-CCAGCAACACAACAACATTT-3′ (forward), 5′-ATCTTCATCAGTTACCAGCTT-3′ (reverse). The primers of CyPA are 5′-CGTCTGCTTCG-AGCTGTTTG-3′ (forward) and 5′-GTAAAA-TGCCCGCAAGTCAA-3′ (reversed).

WESTERN BLOTTING

Astrcoytes (4×10^5 cells/dish) were replated on a 35-mm petri-dish. After treatment, cells were harvested and gently homogenized on ice in RIPA buffer (25 mM Tris–HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, and 0.1% SDS) containing 1 mM PMSF, and proteinase inhibitor cocktail. Protein concentration was determined with a Bio-Rad DC kit. Proteins (50 μg) were separated using 10% SDS–PAGE and transferred to nitrocellulose membrane. The membrane was incubated for 1 h in PBS buffer containing 8% non-fat milk and 0.3% Tween-20. Mob2 protein was identified by incubating the membrane with anti-Mob2 antibody (1:2,000) for 1 h, followed by HRP-conjugated secondary antibody (1:2,000) and ECL solution.

LENTIVIRUS-MEDIATED KNOCKDOWN OF Mob2 mRNA EXPRESSION

The shRNA-coding oligonucleotides were designed and cloned into the lentiviral vector pLVRNAi-mU6-EF1-GFP using the single oligonucleotide RNAi technology (Biosettia, San Diego, CA). One oligonucleotide (AAAAGCAGTATAGCACAATCTCATTGGATCCAA-TGA-GATTGTGCTATACTGC) was used to construct shRNA-expressing lentiviral (lenti-shRNA) vector targeting rat Mob2 (lenti-shRNA236-Mob2). Astrocytes (5×10^4 cells/2-cm^2 or 4×10^5 cells/35-mm petri-dish) were transduced by the appropriate amount (150–300 μl virus particles) of lenti-shRNA236-Mob2 in 10% CS-containing DMEM/F-12 medium. After lentivirus particle transduction, the cells were cultured in fresh DMFM/F-12 medium containing 10% CS for another 48 h. The cultures were then treated with dbcAMP in serum-free DMEM/F12 medium for 24 h. The knockdown efficiency of Mob2 mRNA and protein expression by shRNA236-Mob2 in dbcAMP-treated astrocytes was determined by Q-PCR using the specific Mob2 primers as indicated above and by Western blot. The cultures were then subjected to F-actin staining. The GFP$^+$-cells were observed under a Nikon E800 fluorescence microscope equipped with a cooling CCD camera system.

Mob2 GENE OVEREXPRESSION IN ASTROCYTES

The construction of the recombinant pcDNA3.1 expression vector encoded by mouse Mob2 (pMob2) was prepared as described previously [Lin et al., 2011]. Astrocytes (5×10^4 cells/2-cm^2 or 4×10^5 cells/35-mm petri-dish) were transduced with 1 μg of pMob2 and 1 μg of pEGFP-N2 by Lipofectamine 2000 in antibiotic-free DMEM/F-12 medium containing 1% FBS for 6 h.

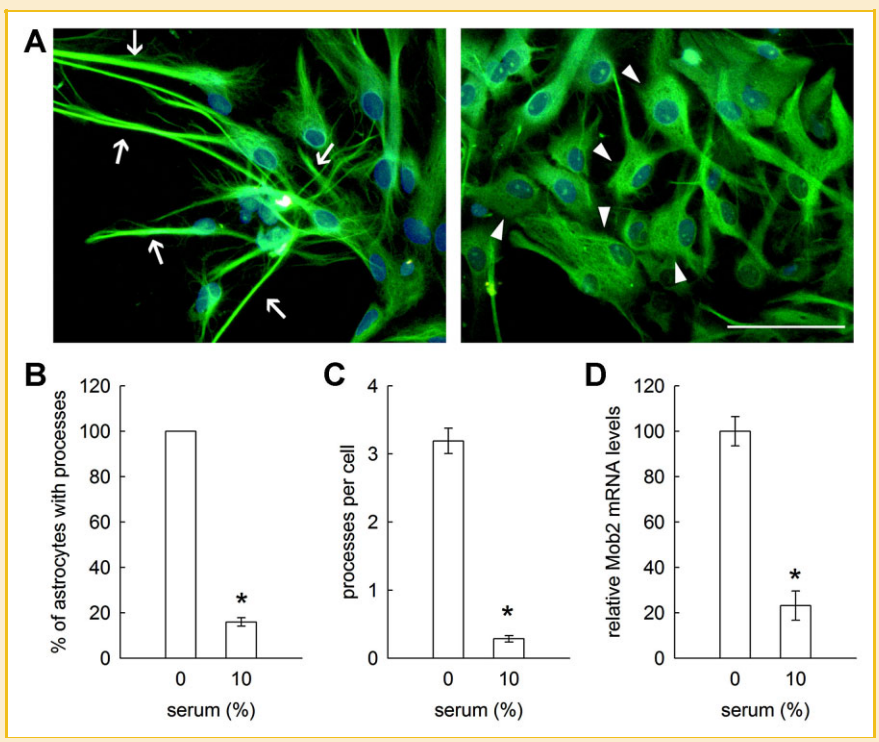

Fig. 1. Serum effect on astrocytic morphological change and Mob2 mRNA expression. Astrocytes were cultured in the presence or absence of 10% CS for 24 h, and then subjected to immunostaining for GFAP (A). GFAP-immunostained stellate processes are indicated by arrows. Representative astrocytes with a flat polygonal shape are indicated by arrowheads. The quantification of astrocytes with stellate processes (B) was performed as described in Materials and Methods Section. Moreover, the number of stellate processes per cell was measured (C). Mob2 mRNA expression was analyzed using Q-PCR method (D). Data are presented as means ± SEM of three independent experiments. Scale bar in A, 50 μm. $^*P < 0.05$ compared with serum-free control. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

The culture medium was replaced with fresh 10% FBS-containing medium for 42 h. The cells were fixed in 4% paraformaldehyde for 10 min, and counterstained in PBS containing 0.1% DAPI for 2 min. We obtained nearly 6% transduction efficiency by determining GFP^+-cells in the culture. GFP fluorescence and DAPI staining were visualized under a Nikon E800 fluorescence microscope equipped with a cooling CCD camera system. The resulting images were captured at 40× magnifications, and five images (1–2 GFP^+-cells per image) from each culture were randomly selected. The total process length per stellate cell with GFP expression was determined using Image J program as described above.

STATISTICAL ANALYSIS

Statistical significance of differences between groups of data was evaluated using two-tailed unpaired Student's *t*-test for quantitative data. In all comparisons, differences were considered statistically significant at $P < 0.05$.

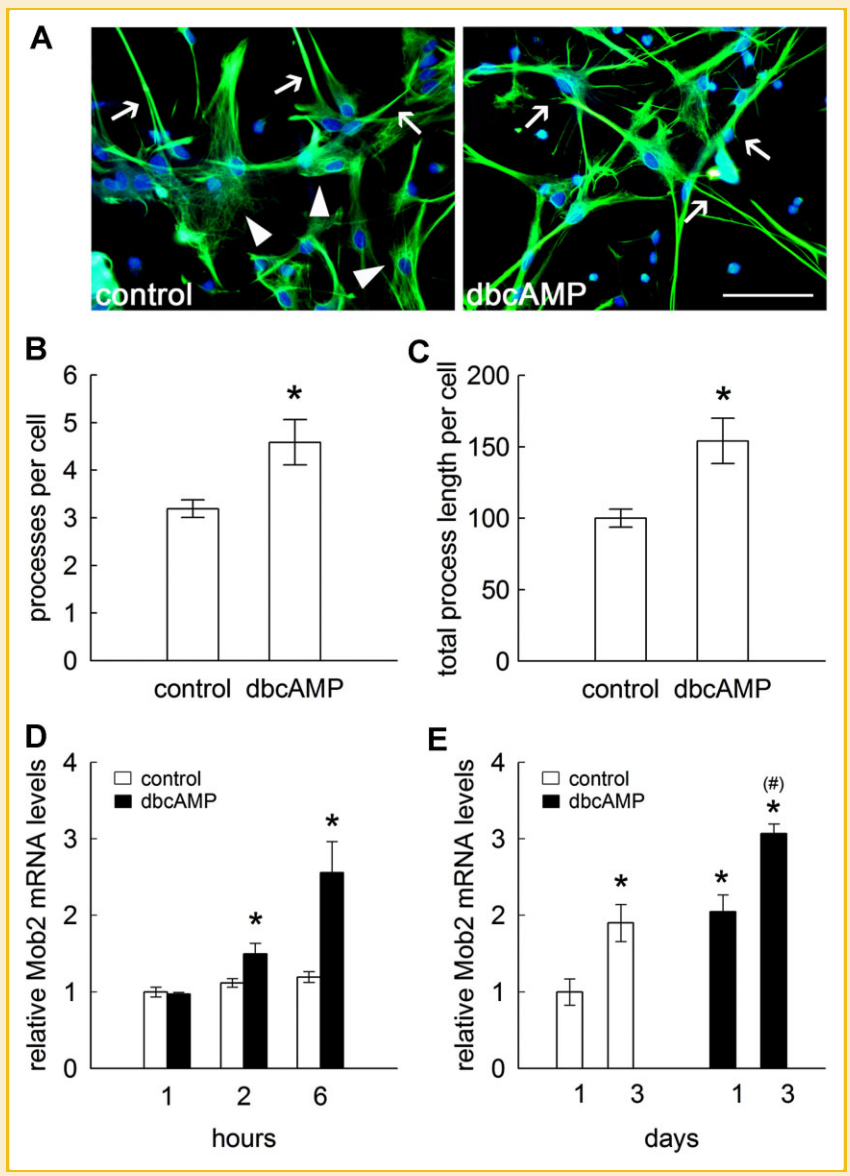

Fig. 2. Enhanced stellation and Mob2 mRNA expression in astrocytes treated by dbcAMP. Astrocytes were treated with or without dbcAMP (150 μM) in serum-free medium for 24 h. Through GFAP immunostaining, astrocytes with long stellate processes (arrows) were mainly observed in the presence of dbcAMP, although few flat polygonal astrocytes (arrowheads) remained to be seen (A). The number of the processes per stellate cell (B) and total length of the processes per stellate cell (C) were measured. Moreover, after treatment with dbcAMP (150 μM) at indicated time points (D,E) in serum-free medium, the cultures were subjected to Q-PCR for the measurement of Mob2 mRNA expression. Data are presented as means ± SEM of at least three independent experiments. $^*P < 0.05$ compared with control (B,C), 1-h control (D) or 1-day control (E). Scale bar in A, 50 μm. $^\#P < 0.05$ versus 1-day treatment with dbcAMP. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

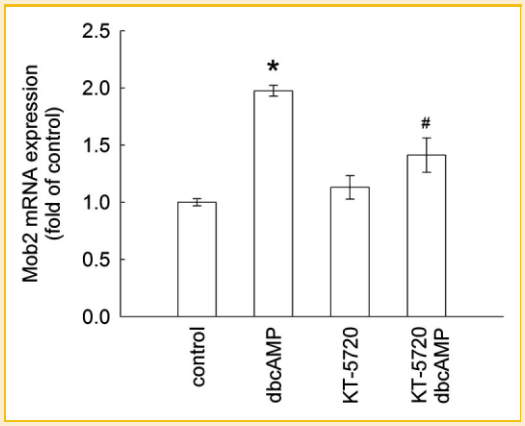

Fig. 3. Blockade of dbcAMP effect on increased Mob2 mRNA expression in astrocytes by KT-5720. Astrocytes were pretreated with the PKA inhibitor KT-5720 (100 nM) in serum-free medium for 30 min. The cultures were exposed to 150 μM of dbcAMP for 6 h, and then subjected to Q-PCR assay for Mob2 mRNA levels. Data are presented as means ± SEM of three independent experiments. $^{*}P < 0.05$ compared with control. $^{\#}P < 0.05$ compared with culture exposed to treatment with dbcAMP alone.

RESULTS

SERUM-INDUCED REDUCTION IN ASTROCYTIC STELLATION AND Mob2 mRNA EXPRESSION

Astrocytes mostly showed an extending process-bearing morphology in the culture without the supply of serum (Fig. 1A, arrows). However, the process-bearing astrocytes were eliminated in serum-containing medium (Fig. 1B), and most of cells displayed a flat and polygonal shape (Fig. 1A, arrowheads). Moreover, the number of astrocytic processes was reduced in serum supplied culture medium (Fig. 1C). Examination of Mob2 mRNA levels using Q-PCR indicated that Mob2 mRNA levels in astrocytes were significantly reduced under serum-containing culture condition (Fig. 1D), suggesting that the decline in astrocytic processes was associated with the downregulation of Mob2 mRNA.

UPREGULATION OF Mob2 EXPRESSION IN ASTROCYTES BY TREATMENT WITH dbcAMP AND PACAP

Stimulation of astrocytes with cAMP analogues in serum-free medium can induce their stellation [Goldman and Abramson, 1990;

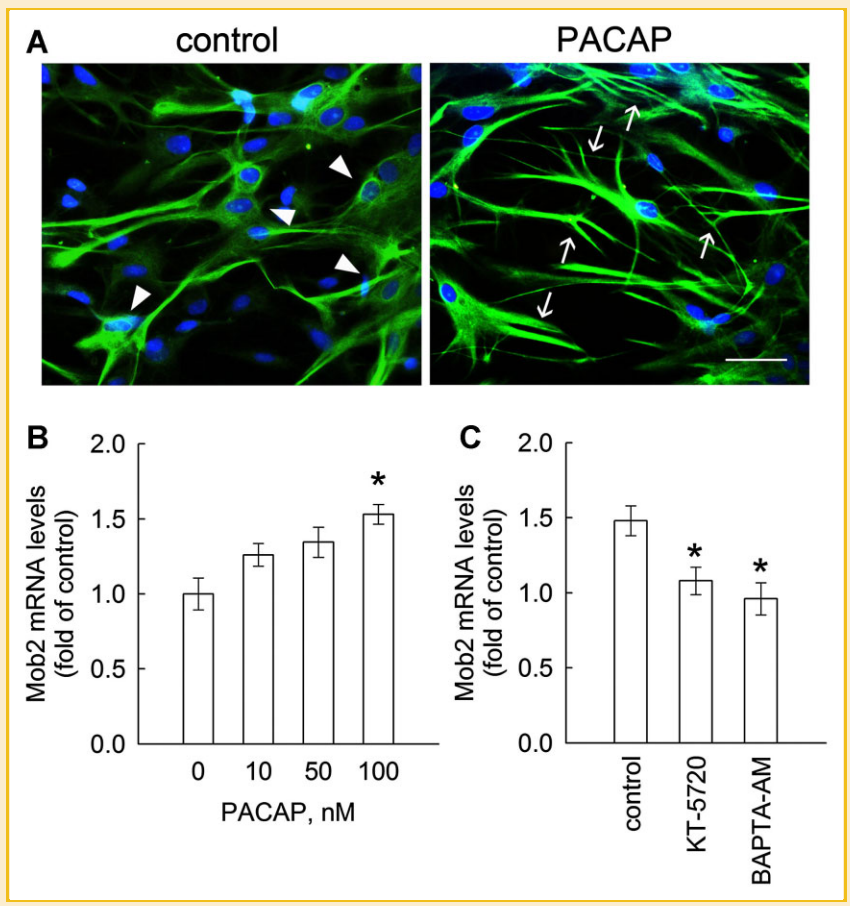

Fig. 4. Upregulation of Mob2 expression in PACAP-induced stellate astrocytes. Astrocytes were treated 100 nM of PACAP in serum-free medium for 24 h, and subjected to immunofluorescence for GFAP (A). Arrows are representative of the stellate processes of astrocytes treated with PACAP, while flat astrocytes are indicated as arrowheads. In addition, astrocytes were exposed to PACAP at the indicated concentrations for 24 h, and then subjected to Q-PCR assay for measurement of Mob2 mRNA levels (B). Alternatively, astrocytes were pretreated with the PKA inhibitor KT-5720 (100 nM) and the Ca^{2+} chelator BAPTA-AM (10 μM) for 30 min (C), followed by exposure to PACAP (100 nM) for another 6 h. The cultures were subject to Q-PCR for measurement of Mob2 mRNA expression. Data shown in B are presented as means ± SEM of three independent experiments. Scale bar in A, 50 μm. $^{*}P < 0.05$ compared with control. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

Won and Oh, 2000; Schmidt-Ott et al., 2001; Vallejo and Vallejo, 2002]. To examine if Mob2 mRNA expression was regulated by dbcAMP, astrocytes were treated with dbcAMP in serum-free medium for 24 h, and then subjected to GFAP immunostaining. It was noted that there were process-bearing astrocytes (Fig. 2A, arrows) and astrocytes with a flat polygonal shape (Fig. 2A, arrowheads) in the control culture. Moreover, dbcAMP enhanced multiple extending processes in astrocytes (Fig. 2A, arrows). As shown in Figure 2B,C, exposure to dbcAMP for 24 h caused an increase in the number of astrocytic processes and the total process length per stellate cell. Furthermore, Q-PCR analysis indicated that Mob2 mRNA expression was increased time-dependently by the addition of dbcAMP (Fig. 2D). Moreover, Mob2 mRNA levels in the culture treated with dbcAMP for 3 days was significantly increased when compared to that detected in the culture by exposure to dbcAMP for 24 h (Fig. 2E). The results suggest that cAMP-dependent signaling regulated Mob2 mRNA expression. To determine if PKA was involved in cAMP-dependent regulation of Mob2 mRNA expression, astrocytes were pretreated with the PKA inhibitor KT-5720 before exposure to dbcAMP. Indeed, pretreatment of dbcAMP-primed astrocytes with KT-5720 caused significant reduction in Mob2 mRNA expression (Fig. 3). These results reveal that Mob2 mRNA expression in primary astrocytes was regulated by the PKA-dependent signal pathway.

PACAP functions as an inducer of cAMP-dependent pathway to stimulate astrocytic differentiation of cortical progenitor cells [Vallejo and Vallejo, 2002]. Here, we found that exposure to PACAP for 24 h effectively induced stellate astrocytic process branching (Fig. 4A, arrows). Moreover, treatment with PACAP for 24 h increased Mob2 mRNA expression in astrocytes (Fig. 4B). PACAP has been known to increase intracellular cAMP through the activation of adenylyl cyclase, and provoked Ca^{2+} mobilization from IP3-sensitive pools in astrocytes [Tatsuno et al., 1996; Masmoudi et al., 2003]. We further observed that PACAP effect on the regulation of Mob2 mRNA expression was blocked by pretreatment with KT-5720 and intracellular Ca^{2+} chelator BAPTA-AM (Fig. 4C). These results verified that Mob2 expression in stellate astrocytes was regulated by cAMP/PKA-dependent pathway. In consistence with the upregulation of Mob2 mRNA expression, Mob2 protein levels in astrocytes were also found to be elevated by dbcAMP and PACAP for 24 h when compared to that detected in serum-free control culture (Fig. 5). In contrast, there was a low level of Mob2 proteins when astrocytes were cultured in serum containing medium (Fig. 5).

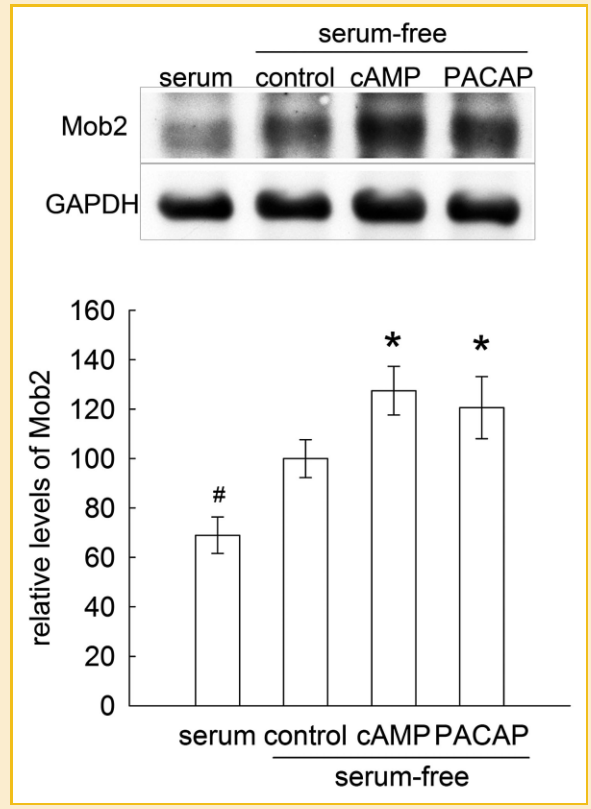

Fig. 5. Mob2 protein expression increased in astrocytes treated by dbcAMP and PACAP. Astrocytes were cultured in serum-containing medium for 24 h, or the cells were treated in serum-free medium with dbcAMP (150 μM) and PACAP (100 nM) for 24 h. The cultures were subjected to Western blot analysis for the examination of Mob2 protein levels (top panel). The same blots were reprobed with anti-GAPDH as a loading control. Data are presented as means ± SEM of three independent experiments. *P (increased) or #P (reduced) < 0.05 compared with serum-free control.

Mob2 PROMOTES ASTROCYTIC STELLATION

Similar to the observation shown in Figure 1A, F-actin immunostaining indicated that astrocytes cultured in serum-free medium expressed longer processes (Fig. 6A, arrows), while astrocytes in serum-containing medium had a flat polygonal shape (Fig. 6A, arrowheads). Stellate astrocytes enhanced by dbcAMP developed 2–3 elongated processes from cell soma (Fig. 6A,C, arrows). Treatment with PACAP also induced web-linking interaction between the processes of stellate astrocytes (Fig. 6A, arrows). Moreover, pre-exposure with KT-5720 suppressed dbcAMP- and PACAP-induced stellation (Fig. 6B, arrowheads). F-actin immunofluorescence further confirmed that the morphology of dbcAMP- and PACAP-treated astrocytes became flat polygonal after pre-treatment with KT-5720 (Fig. 6C, arrowheads). In addition, KT-5720 significantly reduced the number of stellate astrocytes in the presence of dbcAMP or PACAP (Fig. 6D).

To determine the role of Mob2 in astrocytic stellation, lentivirus-mediated expression of shRNAs against Mob2 (sh-Mob2) was applied to inhibit Mob2 expression in dbcAMP-treated astrocytes. Mob2 gene silencing efficiency in dbcAMP-treated astrocytes transduced by lenti-sh-Mob2 used in the study was first to be verified (Fig. 7A). Mob2 protein production in dbcAMP-treated astrocytes was also significantly inhibited by lenti-sh-Mob2 (Fig. 7B). Furthermore, F-actin immunostaining indicated that dbcAMP-treated astrocytes displayed less stellate after transduction with lenti-sh-Mob2 (Fig. 7C, arrowheads), when compared to those observed in dbcAMP-treated culture transduced by control lentivirus particles (Fig. 7C, arrows). Transduction of sh-Mob2 also significantly reduced the number of the extended process-bearing astrocytes in the presence of dbcAMP and PACAP (Fig. 7D).

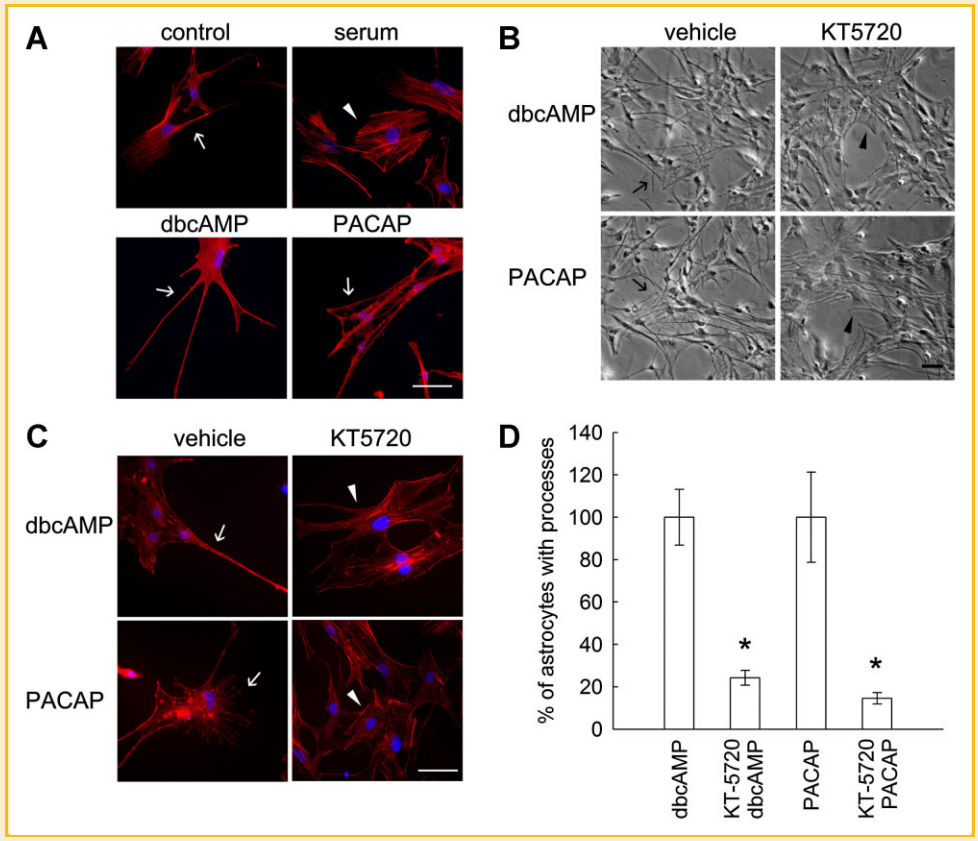

Fig. 6. Inhibition of dbcAMP- and PACAP-induced astrocytic stellation by KT-5720. Astrocytes were cultured in serum-free medium (control), 10% serum-containing medium (serum), or in serum-free medium with dbcAMP (150 μM) and PACAP (100 nM) for 24 h. The cells were then subjected to Texas red-conjugated phalloidin for F-actin (A). Alternatively, astrocytes were pretreated with KT-5720 (100 nM) for 30 min, and then exposure to dbcAMP and PACAP at the indicated concentration for 24 h. The morphology of astrocytes was observed under phase-contrast microscopy (B). The cultures were incubated with Texas red-conjugated phalloidin for F-actin staining (C). Moreover, the number of astrocytes with stellate processes from at least 4 random fields was quantified as the ratio of stellate astrocytes to total number of cells per field (D). Values represent the percentage of stellate astrocytes in the treated group over the relative control culture (dbcAMP alone or PACAP alone). Arrows in A–C indicate stellate processes of astrocytes. Arrowheads in A–C indicate the cells with a flat polygonal shape. Scale bar in A–C, 50 μm. Data shown in D are presented as means ± SEM of three independent experiments. $^{*}P < 0.05$ compared with dbcAMP alone or PACAP alone. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

In addition, overexpression of astrocytes with Mob2 gene under a serum-containing culture condition was performed by co-transduction of astrocytes with pMob2 and pEGFP-N2. In the presence of serum, GFP^{+}-cells cotransduced by control pcDNA displayed a flat polygonal shape (Fig. 8A, arrowheads), and stellate cells were hardly observed in the control culture. However, GFP^{+}-cells with Mob2 gene transduction showed a stellate form (Fig. 8A, arrows). The quantitative analysis indicated that the total length of the processes per cell was significantly increased in GFP^{+}-cells with Mob2 gene transduction compared to that with control plasmid transduction (Fig. 8B). The results demonstrate that Mob2 upregulation contributes to the induction of astrocytic stellation.

DISCUSSION

Astrocytic stellation is associated with the change of epithelial-like cell shape to the process-bearing form. Mob2 has been recently reported to involve neurite formation of Neuro2A cells [Lin et al., 2011]. Here, we demonstrate that the upregulation of Mob2 expression in astrocytes by dbcAMP and PACAP is along with astrocytic stellation. This increased expression of Mob2 mRNA in astrocytes is cAMP/PKA-dependent. Moreover, the cAMP-induced stellation of astrocytes can be abolished by knockdown of Mob2 expression, indicating that Mob2 is a regulator of astrocytic stellation.

The study has revealed that Mob2 is localized at the initial sprouting and branching points of the neurites growing from Neuro2A cell soma [Lin et al., 2011]. Moreover, the downregulation of Mob2 in Neuro2A cells results in the failure of neurite sprouting [Lin et al., 2011]. Since actin filaments could play the role in initial sprouting of neurites, it has been suggested that Mob2 mediates the rearrangement of actin cytoskeleton to induce neurite sprouting and branching [Lin et al., 2011]. The morphological transformation of astrocytes is relevant to the rearrangement of GFAP filaments and actin proteins [Ridet et al., 1997; Fenteany and Zhu, 2003]. In consistence with the findings from others [Goldman and Abramson,

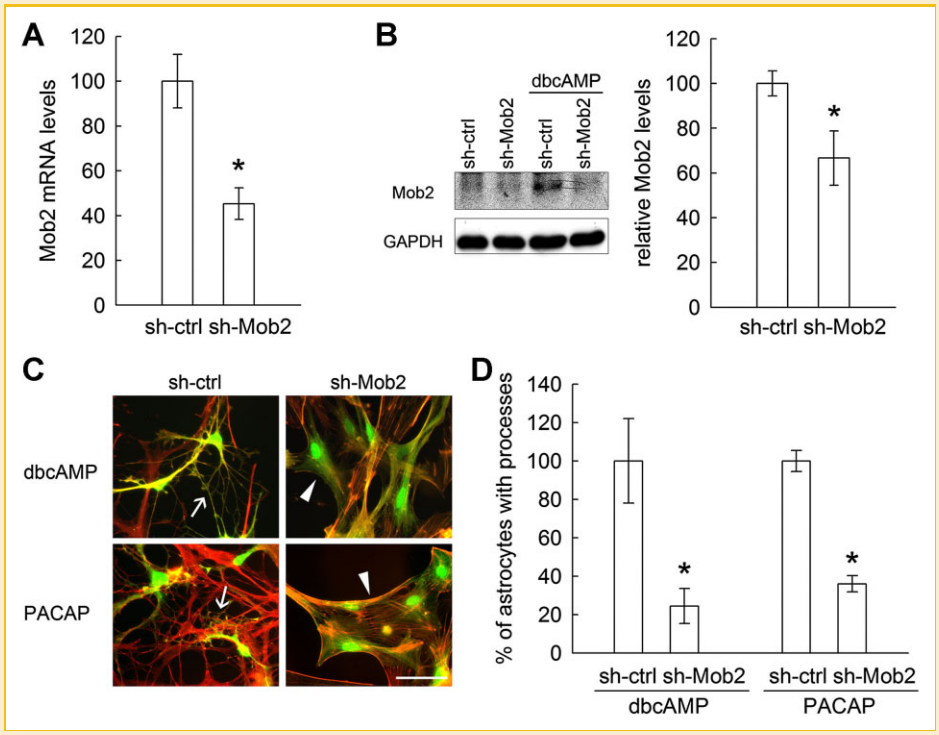

Fig. 7. Reduction of dbcAMP- and PACAP-induced astrocytic stellation by knockdown of Mob2 expression. A: Astrocytes were transduced for 24 h by control lentivirus (sh-ctrl) and by lentivirus expressing Mob2-shRNA (sh-Mob2). After a 48-h expression in serum-containing medium, the cultures were treated with or without dbcAMP (150 μM) in serum-free medium for 24 h. The cultures were subjected to Q-PCR assay for measurement of Mob2 mRNA levels. The results were normalized with CyPA, and expressed as the percentage of Mob2/CyPA with control (sh-ctrl). B: After astrocytes were transduced by sh-ctrl or sh-Mob2 and followed by a 48 h expression in serum-containing medium, the transduced astrocytes were treated with dbcAMP for another 24 h in serum-free medium. The cultures were subjected to Western blot analysis for the examination of Mob2 protein expression. The same Western blots were reprobed with anti-GAPDH as a loading control. Quantification of protein levels were performed by densitometry and presented as a percentage of the Mob2 protein level compared with the control group (sh-ctrl). C: Astrocytes were transducted by sh-ctrl or sh-Mob2 for 24 h and cultured by a 48-h expression in serum-containing medium. The cultures were exposed to dbcAMP (150 μM) and PACAP (100 nM) in the serum-free medium for 24 h, and then subjected to F-actin staining. GFP^+-astrocytes transduced by sh-ctrl displayed a stellate shape in the presence of dbcAMP or PACAP (arrows). GFP^+-astrocytes infected by sh-Mob2 (green) showed a flat polygonal form (arrowheads). D: After exposure to dbcAMP (150 μM) and PACAP (100 nM) for 24 h, stellate astrocytes from at least 4 random fields per culture were quantified by counting the stellate GFP^+-astrocytes with transduction of sh-ctrl or sh-Mob2. Values represent as a percentage of stellate astrocytes in the treated group over the control culture. Data shown in A, B, and D are presented as means ± SEM of three independent experiments. Scale bar in C, 50 μm. $^*P < 0.05$ compared with control (sh-ctrl). [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

1990; Baorto et al., 1992; Vallejo and Vallejo, 2002; Perez et al., 2005], through GFAP and F-actin immunostaining, we found that exposure to dbcAMP or PACAP promoted astrocytic stellation. Our further findings indicate that the change in the cell shape of astrocytes was concomitant to the upregulation and Mob2 mRNA expression. In conjunction with the observation showing that dbcAMP-induced stellation of astrocytes was suppressed by knockdown of Mob2 expression, we suggest that an increase in astrocytic Mob2 expression can induce actin cytoskeleton rearrangement to promote astrocytic stellation.

The molecular mechanism for the regulation of Mob2 expression in the cells is still unknown. This is possibly due to no information regarding the promoter sequence of Mob2. Our results that the application of dbcAMP and PACAP caused significant increase in Mob2 mRNA expression in astrocytes, suggesting that the cAMP-dependent pathway regulates Mob2 gene expression. Indeed, pretreatment with KT-5720, the inhibitor of the cAMP downstream effector PKA, blocked the effect of dbcAMP and PACAP on the upregulation of Mob2 mRNA expression in astrocytes. The data provide the first evidence of cAMP/PKA-induced upregulation of Mob2 in stellate astrocytes. On the other hand, PACAP has been shown to increase in intracellular Ca^{2+} levels in astrocytes [Tatsuno et al., 1996; Grimaldi and Cavallaro, 1999; Masmoudi et al., 2003]. The addition of the chelator of intracellular Ca^{2+}, BAPTA-AM, can suppress PACAP-induced increase in Mob2 mRNA expression in astrocytes. Nevertheless, the involvement of Ca^{2+}-dependent pathway in Mob2 mRNA expression remains to be further confirmed.

Taken together, our study indicates that Mob2 involves the induction of astrocytic stellation via triggering actin cytoskeleton rearrangement. In addition, we elucidate that the cAMP signaling pathway plays an important role in the regulation of Mob2 mRNA expression. The characterization of microenvironmental clues to regulate Mob2 expression may provide useful insight in defining molecular mechanism underlying astrocytic stellation during CNS development.

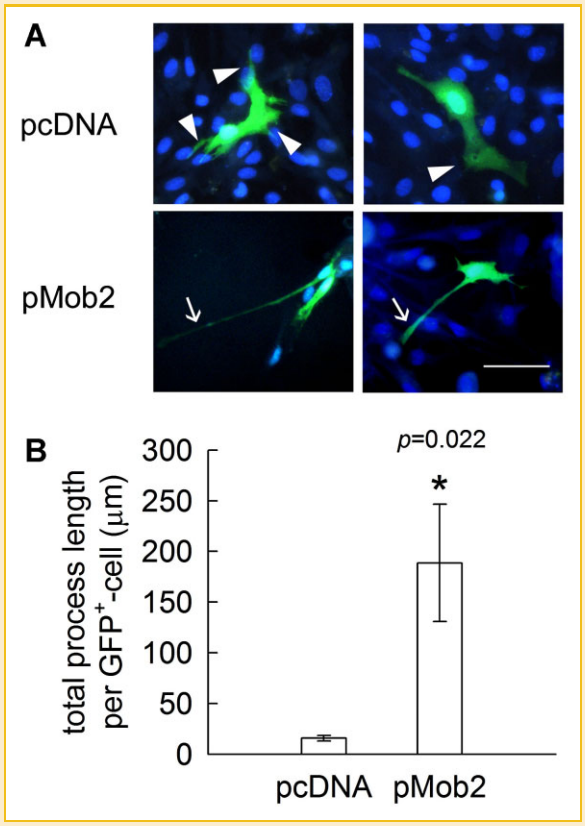

Fig. 8. Improved stellation of astrocytes by Mob2 overexpression. Astrocytes were cotransduced by pMob2 (or control pcDNA) with pEGFP-N2 as described in Materials and Methods. The cells were then fixed and visualized under a fluorescence microscope. GFP^+-astrocytes with a flat polygondal shape in the pcDNA-control culture are indicated by arrowheads (A). GFP^+-astrocytes having extending processes observed in the pMob2-transduced culture are indicated by arrows (A). Note that no GFP^+-stellate cell was observed in the culture transduced by control pcDNA. The total length of processes per GFP^+-cell with pcDNA or pMob2 transduction was measured from five random fields (5–8 GFP^+-cells) per culture (B). Data are presented as means ± SEM of three experiments. $^*P < 0.05$ compared with control. Scale bar in A, 50 μm. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

ACKNOWLEDGMENTS

The authors thank Ms. Ti-Ju Chan for technical support. This work is supported by the National Science Council, Taiwan (NSC 95-2311-B-006-004 and NSC-99-2811-B-006-058).

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