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Nuclear export of OLIG2 in neural stem cells is essential for ciliary neurotrophic factor–induced astrocyte differentiation

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Neural stem cell (NSC) differentiation is precisely controlled by a network of transcription factors, which themselves are regulated by extracellular signals (Bertrand, N., D.S. Castro, and F. Guillemot. 2002. *Nat. Rev. Neurosci.* 3:517–530; Shirasaki, R., and S.L. Pfaff. 2002. *Annu. Rev. Neurosci.* 25:251–281). One way that the activity of such transcription factors is controlled is by the regulation of their movement between the cytosol and nucleus (Vandromme, M., C. Gauthier-Rouviere, N. Lamb, and A. Fernandez. 1996. *Trends Biochem. Sci.* 21:59–64; Lei, E.P., and P.A. Silver. 2002. *Dev. Cell.* 2:261–272). Here we show that the basic helix–loop–helix transcription factor OLIG2, which has been shown to be required for motor neuron and oligodendrocyte development, is found in the cytoplasm, but not the nucleus, of astrocytes in culture and of a subset of astrocytes in the subventricular zone. We demonstrate that the accumulation of OLIG2 in the nucleus of NSCs blocks the CNTF-induced astrocyte differentiation and that the translocation of OLIG2 to the cytoplasm is promoted by activated AKT. We propose that the AKT-stimulated export of OLIG2 from the nucleus of NSCs is essential for the astrocyte differentiation.

Introduction

OLIG2 is a basic helix–loop–helix (bHLH) transcription factor required for oligodendrocyte development (Lu et al., 2000; Takebayashi et al., 2000; Zhou et al., 2000, 2001). OLIG2 also blocks astrocyte differentiation, by inhibiting complex formation between STAT3 and the transcription coactivator p300 (Lu et al., 2002; Takebayashi et al., 2002; Zhou and Anderson 2002; Gabay et al., 2003; Fukuda et al., 2004). Despite the crucial role of OLIG2 in glial fate decision, little is known about how OLIG2 itself is regulated during glial development. Previous findings have suggested that when neural stem cells (NSCs) begin to differentiate into glial–fibrillary–acidic–protein (GFAP)-positive astrocytes in culture, OLIG2 disappears from the nucleus and is sometimes detected in the cytoplasm (Fukuda et al., 2004), raising the possibility that the translocation of OLIG2 from the nucleus to cytoplasm might be crucial for astrocyte differentiation.

Here we show that OLIG2 is detected in the cytoplasm of GFAP-positive astrocytes in the subventricular zone (SVZ) of adult mouse brain, which have been shown to act as NSCs that give rise to both neurons and glia. We demonstrate that Leptomycin B (LMB), which is a specific inhibitor of CRM1-dependent nuclear export (Fornerod et al., 1997; Yoshida and Horinouchi, 1999), blocks both the export of OLIG2 from the nucleus and astrocyte differentiation induced by ciliary neurotrophic factor (CNTF). Moreover, we show that an OLIG2 mutant lacking a putative CRM1 binding site is deficient in both CNTF-induced nuclear export and astrocyte differentiation. Finally, we show that AKT, which is activated by CNTF stimulation, phosphorylates OLIG2 in vitro and that an OLIG2 mutant lacking an AKT phosphorylation site is also deficient in both CNTF-induced nuclear export and astrocyte differentiation. These findings suggest that AKT-dependent nuclear export of OLIG2 plays a crucial part in astrocyte differentiation.

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Abbreviations used in this paper: bFGF, basic FGF; bHLH, basic helix-loop-helix; CNTF, ciliary neurotrophic factor; GFAP, glial fibrillary acidic protein; LMB, leptomycin B; NES, nuclear export signal; NF, neurofilament; NSC, neural stem cell; PI, propidium iodide; SVZ, subventricular zone; VZ, ventricular zone.

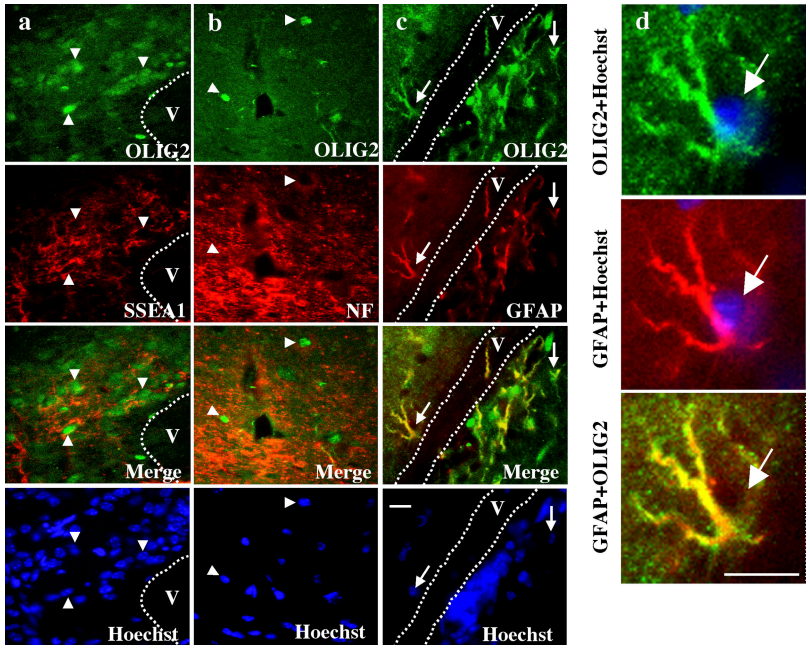

Figure 1. **Evidence of OLIG2 translocation from the nucleus of astrocytes in the SVZ of adult mouse brain.** Brain sections of adult mice were labeled for both OLIG2 (green) and a neural marker (red); (a) SSEA1, (b) NF, or (c and d) GFAP. The sections were counterstained with Hoechst 33342 (blue). Arrowheads and arrows show OLIG2-positive cells in the nucleus and in the cytoplasm, respectively. V shows ventricle. Bars, 10 μm.

Results and discussion

Export of OLIG2 from the nucleus is essential for astrocyte differentiation

We first investigated whether OLIG2 localized in the cytoplasm of astrocyte in vivo. We immunolabeled sections of either newborn or adult mouse brain for both OLIG2 and a neural marker—SSEA1 for NSCs, neurofilament (NF) for neurons, or GFAP for astrocytes. In the SVZ of the adult brain, 44% of the cells with OLIG2 in the nucleus stained for SSEA1, and 38% stained for NF (Fig. 1, a and b). None of GFAP-positive cells had OLIG2 in the nucleus, although 20% of them had OLIG2 in the cytoplasm (Fig. 1 c). In the ventricular zone (VZ) of the newborn brain, we also found that 57% of the cells with OLIG2 in the nucleus stained for SSEA1, and 41% stained for NF; none of GFAP-positive cells had OLIG2 in the nucleus, although 4% of them had OLIG2 in the cytoplasm (unpublished data). It seems, therefore, that, in the VZ and SVZ, OLIG2 is mainly in the nucleus of both NSCs and neurons, by contrast, the few astrocytes contained OLIG2 exclusively in their cytoplasm.

We investigated further the relationship between OLIG2 localization and astrocyte differentiation in culture. We prepared NSCs from embryonic mouse telencephalon and expanded them in both basic FGF (bFGF) and EGF for 2 wk. Over 90% of the NSCs were OLIG2-positive by 2 wk (Fukuda et al., 2004). To induce astrocyte differentiation, we withdrew both the bFGF and EGF and added CNTF (Fukuda et al., 2004). We used LMB to block active export from the nucleus. After 2 d in CNTF, over 50% of the cells differentiated into GFAP-positive astrocytes; none of the GFAP-positive cells had OLIG2 in their nucleus (Fig. 2 a), but 10% had OLIG2 in their cytoplasm (Fig. 2, a and b). By contrast, the GFAP-negative cells still had OLIG2 in their nucleus (Fig. 2 a), and these cells stained for Nestin that is a marker for NSCs (Fig. 2 d). After 4 d, 90% of the cells were GFAP-positive and OLIG2-negative (not shown). After 2 d in CNTF plus LMB, compared with the NSCs cultured in CNTF alone for 2 d, NSC proliferation had significantly decreased, the cell bodies had become larger, over 95% of the cells were GFAP-negative and had OLIG2 in the nucleus (Fig. 2 c). These findings suggest that CNTF-induced astrocyte differentiation is associated with CRM1-dependent translocation of OLIG2 from the nucleus into the cytoplasm.

Because it was shown previously that STAT3 accumulates in the nucleus when NSCs are induced by CNTF/LIF to express GFAP (Bonni et al., 1997; Nakashima et al., 1999), we examined STAT3 localization in NSCs cultured in either

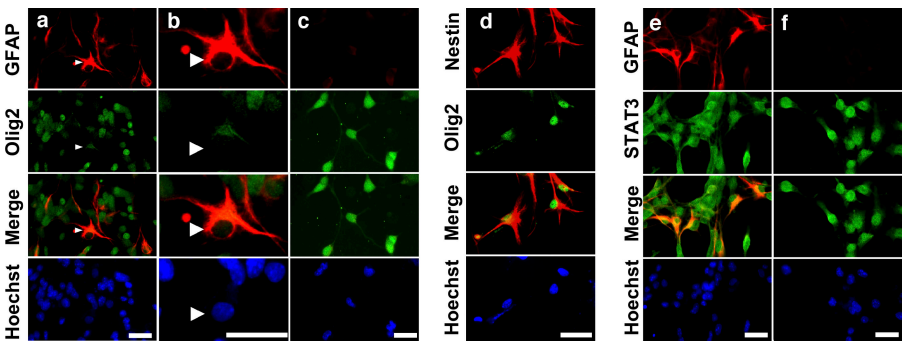

Figure 2. **Translocation of nuclear proteins from the nucleus is required for CNTF-induced astrocyte differentiation.** (a, b, d, and e) NSCs cultured in either CNTF alone or (c and f) CNTF plus LMB for 2 d were labeled for OLIG2 (green) and (a–c) GFAP (red), OLIG2 (green), and (d) Nestin (red), or (e and f) STAT3 (green) and GFAP (red). The cells were counterstained with Hoechst 33342 (blue). Arrowhead shows OLIG2-positive cells in the cytoplasm. Bar, 25 μm.

CNTF alone or CNTF plus LMB. After 2 d in CNTF alone, STAT3 was mainly in the nucleus, and >50% of the cells stained for GFAP, and many looked like astrocytes (Fig. 2 e). By contrast, after 2 d in CNTF plus LMB, STAT3 strongly accumulated in the nucleus, but none of the cells were GFAP positive and acquired an astrocytic morphology (Fig. 2 f). Thus, nuclear accumulation of STAT3 is apparently not enough to induce astrocyte differentiation; nuclear export of transcriptional repressors such as OLIG2 may also be required.

CNTF-dependent OLIG2 translocation from the nucleus depends on CRM1

Because it seemed likely that OLIG2 export from the nucleus would depend on the nuclear export signal receptor CRM1, we looked for CRM1-binding sites in OLIG2. We found one putative nuclear export signal sequence (NES; 146-LSKIATLLL-154). To examine whether this sequence was a functional NES, we replaced the leucines at 152 and 154 to alanines and examined the localization of the mutant protein in Cos7 cells. When FLAG-tagged wild-type OLIG2 (OLIG2-w) was overexpressed in Cos7 cells, it was diffusely distributed (Fig. 3 a), although in the presence of LMB it accumulated in the nucleus (Fig. 3 b), suggesting that OLIG2 is normally exported from the nucleus by CRM1 in Cos7 cells. By contrast, when FLAG-tagged mutant OLIG2 (OLIG2–NES) was expressed in Cos7 cells, it accumulated in the nucleus even in the absence of LMB (Fig. 3 c), confirming that 146-LSKIATLLL-154 is the relevant CRM1-dependent export signal in OLIG2.

Because it has been shown that enforced expression of OLIG2-w does not block CNTF-induced astrocyte differentiation completely (Fukuda et al., 2004), we examined whether OLIG2–NES would be more effective at blocking CNTF-induced astrocyte differentiation. We overexpressed OLIG2–NES in NSCs, cultured them in the presence of CNTF for 2 d, and then immunolabeled them for GFAP. Whereas >50% of the cells transfected with a control vector and 35% of the cells transfected with OLIG2-w were labeled for GFAP (Fig. 3, d and f; and data not depicted), <10% of the cells transfected with OLIG2–NES were labeled for GFAP (Fig. 3, e and f). Together with the previous findings (Fukuda et al., 2004), these results suggest that CRM1-dependent OLIG2 translocation from the nucleus is crucial for CNTF-induced astrocyte differentiation.

It has been shown that the nuclear corepressor N-CoR, which inhibits astrocyte differentiation, is also exported from the nucleus, following CNTF stimulation (Hermanson et al., 2002), raising the possibility that OLIG2 and N-CoR may be physically associated. When we immunolabeled cultured NSCs with anti N-CoR antibodies, ~10% of the cells showed nuclear staining (Fig. 4 a). CNTF stimulation caused the N-CoR to translocate to the cytoplasm (Fig. 4 b), as reported previously (Hermanson et al., 2002). However, we detected OLIG2 in the nucleus of >90% of the cultured NSCs (Fukuda et al., 2004), making it unlikely that OLIG2 is associated with N-CoR in the cells. To test this conclusion further, we transfected either a control vector expressing GFP or a vector expressing OLIG2–NES, cultured them in

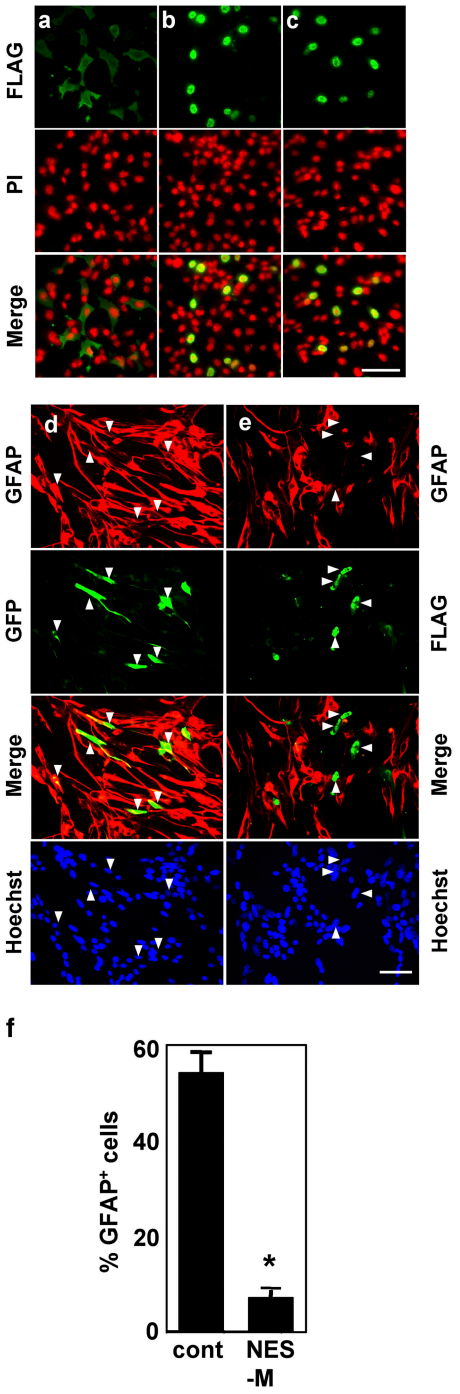

Figure 3. **Nuclear accumulation of OLIG2 blocks astrocyte differentiation.** (a–c) Cos7 cells were transfected with either (a and b) FLAG-tagged OLIG2-w or (c) FLAG-tagged OLIG2–NES. (b) LMB was used to block active export from the nucleus. Transfected OLIG2 was detected with anti-FLAG antibody (green) and the cells were counterstained with PI (red). NSCs were transfected with either (d) the control vector or (e) OLIG2–NES, cultured in CNTF, and labeled for (d) either GFP (green) and GFAP (red) or (e) FLAG (green) and GFAP (red). Arrowheads show the transfected cells. (f) The proportions of GFAP positive cells in transfected cells are shown as the mean ± SD of three cultures. *, $P < 0.01$ by *t* test. Bars: (a–c) 50 μm; (d and e) 25 μm.

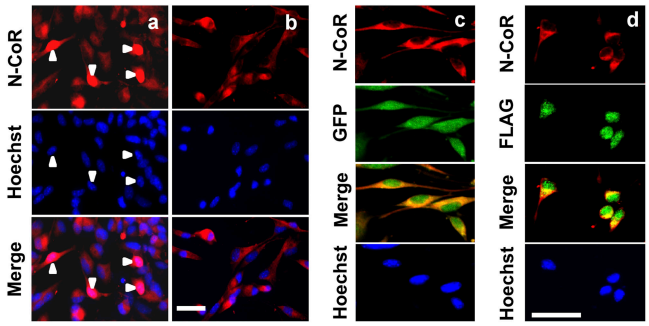

Figure 4. **Localization of OLIG2 and N-CoR is regulated independently.** NSCs were cultured in either (a) bFGF plus EGF or (b) CNTF alone, and then labeled for N-CoR (red). Arrowheads show N-CoR–positive cells in the nuclei. NSCs were transfected with either (c) the control vector or (d) OLIG2–NES, cultured in the presence of CNTF, and then (c) labeled for either GFP (green) and N-CoR (red) or (d) FLAG (green) and N-CoR (red). The cells were counterstained with Hoechst 33342 (blue). Bars: 25 μm.

CNTF for 2 d, and then immunolabeled them for N-CoR and either GFP or FLAG. In the cells transfected with either the control vector or the OLIG2–NES vector, N-CoR was detected only in the cytoplasm (Fig. 4, c and d), whereas OLIG2–NES remained in the nucleus (Fig. 4 d). It seems, therefore, that OLIG2 and N-CoR independently regulate astrocyte differentiation.

CNTF stimulated PI-3 kinase (PI3K)/AKT activation induces OLIG2 translocation from the nucleus

It was shown previously that CNTF can activate PI3K, as well as STAT3 (Alonzi et al., 2001). Moreover, the PI3K inhibitor LY294002 blocks both CNTF-induced astrocyte differentiation and CNTF-induced N-CoR translocation from the nucleus (Hermanson et al., 2002). As we could not detect N-CoR in the nucleus of most of our cultured NSCs (Fig. 4 a), we tested whether PI3K signaling also regulates OLIG2 localization. We cultured NSCs in the presence of either CNTF alone or CNTF plus LY294002 for 3 d and then immunolabeled the cells for both OLIG2 and an astrocyte marker (either GFAP or S100β). In CNTF alone, >70% of NSCs differentiated into GFAP-positive and S100β-positive astrocytes, which had lost OLIG2 from the nucleus (Fig. 5, a and c; and online supplemental material available at http://www.jcb.org/cgi/content/full/jcb.200404104/DC1). 10% of either GFAP-positive or S100β-positive cells still had OLIG2 in the cytoplasm. 20% of the cells retained OLIG2 in their nucleus, and all of them were GFAP negative (Fig. 5 a). By contrast, in the presence of CNTF and LY294002, >90% of the cells were GFAP negative and S100β negative and retained OLIG2 in the nucleus (Fig. 5, b and c; and online supplemental material available at http://www.jcb.org/cgi/content/full/jcb.200404104/DC1). Thus, PI3K signaling in cultured NSCs apparently promotes OLIG2 translocation from the nucleus and is crucial for CNTF-induced astrocyte differentiation.

Because AKT kinase is a major target of PI3K signaling (Hunter, 2000; Schlessinger, 2000), we looked for AKT phosphorylation sites in OLIG2 and found one (26-SKGGSSSGF-34) centered at serine 30. To examine whether AKT phosphorylates OLIG2 directly, we overexpressed either FLAG-tagged OLIG2-w or a FLAG-tagged mutant OLIG2 in which serine 30 was replaced by alanine (OLIG2-S30A) in NSCs, purified the proteins using anti-FLAG antibody, and then used the proteins in an in vitro kinase assay. Whereas OLIG2-w was phosphorylated by AKT, OLIG2-S30A was not, suggesting that serine 30 in OLIG2 is a direct target of AKT (Fig. 5 d).

To investigate the relationship between AKT-dependent OLIG2 phosphorylation and OLIG2 localization, we overexpressed the OLIG2-w or OLIG2-S30A in Cos7 cells and stained them for FLAG. Whereas OLIG2-w was detected mainly in the cytoplasm (Fig. 5 e), OLIG2-S30A was detected mainly in the nucleus (Fig. 5 f), suggesting that the translocation of OLIG2 from the nucleus depends on AKT-dependent phosphorylation of serine 30. We then examined whether OLIG2-S30A can block astrocyte differentiation. We overexpressed OLIG2-S30A in NSCs, cultured them in the presence of CNTF for 2 d, and then labeled them for GFAP. Whereas >50% of the cells transfected with the control vector differentiated into GFAP-positive astrocytes (Fig. 5, g and i), <5% of the cells expressing OLIG2-S30A did so (Fig. 5, h and i), suggesting that AKT-dependent translocation of OLIG2 from the nucleus is essential for CNTF-induced astrocyte differentiation. Interestingly, BMPs also promote astrocyte differentiation, and they can activate both STAT3 and AKT (D'Alessandro et al., 1994; Gross et al., 1996; Ghosh-Choudhury et al., 2002; Rajan et al., 2003).

In the developing spinal cord, OLIG2 is expressed only in the ventral VZ, where motor neurons and oligodendrocytes, but not astrocytes, are generated (Lu et al., 2000; Takebayashi et al., 2000; Zhou et al., 2000). It therefore seems unlikely that the mechanism of nuclear-to-cytoplasm translocation of OLIG2 that we describe here is relevant for astrocyte differentiation in the spinal cord. However, we found that OLIG2 localized in the cytoplasm of GFAP-positive astrocytes in both the VZ of newborn mouse brain and SVZ of adult mouse brain, suggesting that the translocation of OLIG2 from the nucleus might operate in astrocyte differentiation in the brain. Moreover, once VZ/SVZ cells are dissociated and expanded in culture, independently of their origins, they start to express OLIG2, proliferate indefinitely, and generate neurons, astrocytes and oligodendrocytes (Gabay et al., 2003; Fukuda et al., 2004). Recent evidence has also shown that the inhibition of OLIG2 activity in cultured NSCs blocks their proliferation and induces astrocyte differentiation (Hack et al., 2004). Since nuclear accumulation of OLIG2 is crucial to block astrocyte differentiation in cultured NSCs, these findings suggest that translocation of OLIG2 from the nucleus on its own might be sufficient to induce astrocyte differentiation in cultured NSCs.

Such NSCs are an attractive potential source of neural cells for cell therapy, as it has been shown that they can promote functional recovery in various types of CNS damage (Cao et al., 2002; Okano, 2002; Hallbergson et al., 2003). Hence, it will be important to understand the regulation of OLIG2 in NSCs, especially as there is another evidence that oligodendrocyte precursor cells in the adult brain can behave as NSCs (Nunes et al., 2003).

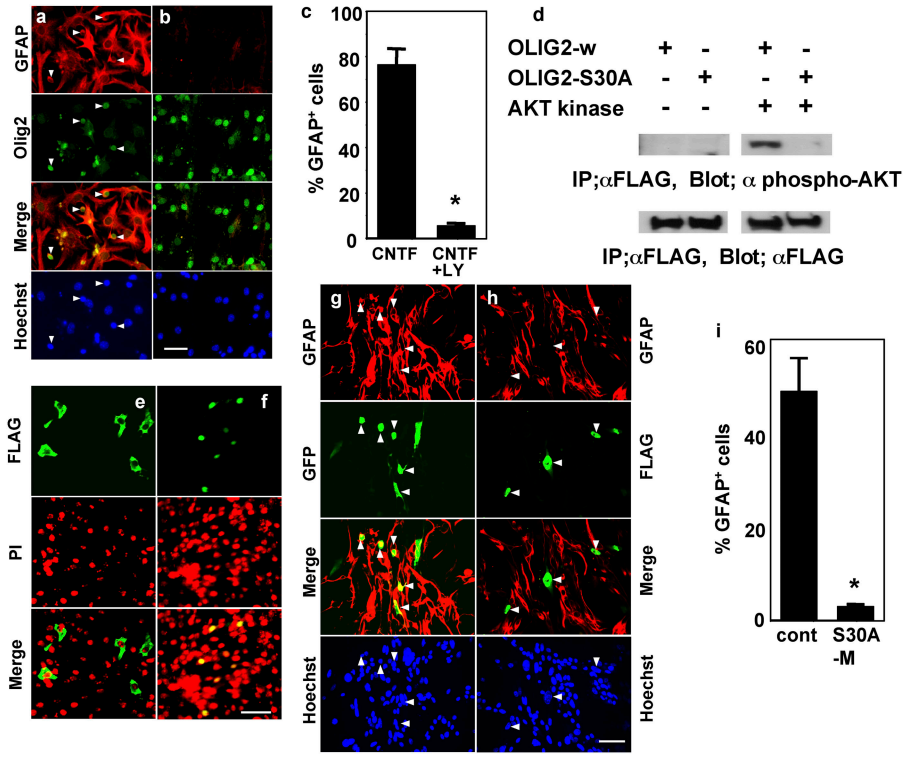

Figure 5. **PI3K/AKT signaling contributes to astrocyte differentiation by inducing nuclear export of OLIG2.** NSCs were cultured in (a) CNTF alone or (b) CNTF plus LY294002, and then stained for OLIG2 (green) and GFAP (red) and with Hoechst 33342 (blue). (c) The proportions of GFAP-positive cells in CNTF alone and CNTF plus LY294002 are shown as the mean ± SD of three cultures. *, $P < 0.001$ by *t* test. (d) Either OLIG2-w or OLIG2-S30A was overexpressed in NSCs, purified using anti-FLAG antibody, and then used for the in vitro AKT kinase reaction. The reaction mixture was analyzed using anti-phospho AKT substrate antibody. Cos7 cells were transfected with (e) FLAG-tagged OLIG2-w or (f) OLIG2-S30A. Transfected OLIG2 was detected with anti-FLAG antibody (green) and the cells were counterstained with PI (red). NSCs were transfected with either (g) the control vector or (h) OLIG2-S30A, cultured in CNTF, and then (g) labeled for either GFP (green) and GFAP (red) or (h) FLAG (green) and GFAP (red). Arrowheads show the transfected cells. (i) The proportions of GFAP-positive cells in transfected cells are shown as the mean ± SD of three cultures. *, $P < 0.01$ by *t* test. Bars: (a and b) 50 μm; (e–h) 25 μm.

Materials and methods

Mice and cell culture

All animal procedures were performed according to the guidelines of the University of Cambridge Centre for Brain Repair. Neuroepithelial cells were isolated from telencephalons of E14.5 CD1 mice and expanded for up to 4 wk in N2-supplemented DME/F-12 containing 10 ng/ml bFGF (Peprotech) and 10 ng/ml EGF (Peprotech) as described previously (Nakashima et al., 1999). Recombinant CNTF (Peprotech) was used for the induction of astrocytes at a concentration of 20 ng/ml. Cos7 cells were maintained in DME supplemented with 10% FCS. In some experiments, LMB (25 ng/ml; Sigma-Aldrich) and LY294002 (50 μM; Calbiochem) were used to inhibit CRM1-dependent nuclear export and PI3 kinase activity, respectively.

Immunochemistry

Newborn and adult CD1 mice (P35w) were killed under deep anesthesia and 10-μm frozen sections of the brain were prepared. Sections were treated with acetone at −20°C for 20 min and 70% ethanol at −20°C for 10 min, blocked with 10% FCS at room temperature for 1 h. Cultured cells were labeled as described previously (Fukuda et al., 2004). The following antibodies were used to detect antigens: mouse anti-NF (1:200; Chemicon), mouse anti-GFAP (1:200; Sigma-Aldrich), rabbit anti-GFAP antibodies (1:500; DakoCytomation), mouse anti-S100β antibody (1:500; Sigma-Aldrich), mouse anti-SSEA1 (1:20; Santa Cruz Biotechnology Corp.), and rabbit anti-OLIG2 antibodies (1:2,000; gift from H. Takebayashi, National Institute for Physiological Sciences, Okazaki, Japan), rabbit anti-STAT3 antibodies (1:200; Santa Cruz Biotechnology Corp.), rabbit anti-N-CoR antibodies (1:200; Santa Cruz Biotechnology Corp.), mouse anti-GFP antibody (1:200; Molecular Probe), mouse anti-FLAG M2 antibody (1:200; Sigma-Aldrich), or their combinations, followed by Alexa-dye–conjugated secondary antibodies (1:200; Molecular Probe) or Texas red–conjugated anti–mouse IgM (1:200; Jackson ImmunoResearch Laboratories), and finally stained with propidium iodide (PI; 1 μg/ml) or Hoechst dye 33342 (1 μg/ml; Molecular Probe) to visualize all nuclei. The stained sections or cells were mounted in Citifluor mounting medium (CitiFluor). The fluorescence images were acquired using an upright fluorescence microscope (model LeiTZDMRD; Leica), a CCD camera (model C4742-95; Hamamatsu), 40× and 60× objectives, and an OpenLab software (Improvision). Images were combined for figures using Adobe Photoshop 5.5.

Transfection

Transfection into NSCs was performed using the Nucleofector according to the supplier's instruction (amaxa). On the following day, the cells were harvested and recultured on poly-L-ornithine (15 μg/ml; Sigma-Aldrich) and fibronectin (1 μg/ml; Invitrogen) -coated eight-well chamber slide (Nunc) as described before (Nakashima et al., 1999). The cells were then induced to differentiate into astrocytes. Transfection into Cos7 cells was performed using the Superfect Transfection Reagent according to the supplier's instruction (QIAGEN). On the following day, the cells were harvested and recultured on coverslips for another 2 d before immunolabeling.

Vector constructs

The expression vector of OLIG2-w (FLAG–OLIG2–pcDNA3) was described before (Fukuda et al., 2004). Mutant form of OLIG2 with the deletion of either NES or AKT phosphorylation site was made using AccuTaq LA DNA polymerase (Sigma-Aldrich) and sets of oligonucleotides containing mutant sequence. The following oligonucleotide DNA primers were synthesized: for the full-length mouse *olig2* cDNA, the 5′ primer was 5′-TTGAATTCATGGACTCGGACGCCAGCCT-3′, and the 3′ primer was 5′-AACTCGAGTCACTTGGCGTCGGAGGTGAG-3′. For a mutant in the NES, the sense primer was 5′-ACGGCGCTGGCGGCGCGAAACTACATC-3′, and the anti-sense primer was 5′-CGCCGCCAGCGCCGTGGCGATCT-TGGA-3′. For a mutant in the AKT phosphorylation site, the sense primer was 5′-AGCAAGGGGGGAGCCAGCAGCGGCTT-3′, and the anti-sense primer was 5′-AAGCCGCTGCTGGCTCCCCCCTTGCT-3′. The mutant cDNAs were fused with FLAG in the pcDNA3 expression vector (Invitrogen) as described previously (Fukuda et al., 2004), resulting in FLAG–OLIG2–NES–pcDNA3 (OLIG2–NES) and FLAG–OLIG2–S30A–pcDNA3 (OLIG2–S30A). pIRES-EGFP (Becton Dickinson) was used as a control vector.

In vitro kinase assay

The in vitro kinase assay was performed following the instructions of the supplier (Cell Signaling Technology). In brief, we transfected 10 μg of either OLIG2-w or OLIG2-S30A to 5×10^6 NSCs using Nucleofector (amaxa). 2 d later, the transfected NSCs were harvested and suspended in cell lysis buffer (Cell Signaling Technology). The cells were sonicated briefly and cell debris was removed by centrifugation. The proteins were immunoprecipitated using anti-FLAG antibody and protein A–Sepharose (Amersham Bioscience),

washed twice in the lysis buffer and three times in the AKT kinase buffer, and were subjected for AKT kinase assay following supplier's instructions (Cell Signaling Technology). The reactions were quenched by addition of SDS-PAGE buffer and analyzed using Western blotting and anti-phospho-AKT substrate antibody (Cell Signaling Technology).

Online supplemental material

Figure S1 shows that PI3K inhibitor LY294002 blocks both S100β expression and nuclear export of OLIG2 induced by CNTF. Online supplemental materials are available at http://www.jcb.org/cgi/content/full/jcb.200404104/DC1.

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