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# C-JUN N-TERMINAL KINASE CONTROLS A NEGATIVE LOOP IN THE REGULATION OF GLIAL FIBRILLARY ACIDIC PROTEIN EXPRESSION BY RETINOIC ACID

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**Abstract—Glial fibrillary acidic protein (GFAP) is a protein widely used as a molecular marker for astroglial differentiation and mature astrocytes. We and others have shown previously that retinoic acid and specific cytokines induce the expression of GFAP in neural precursor cells by activating the phosphatidylinositol-4,5-bisphosphate-3-kinase (PI3K) phosphorylation pathway. Here, we extend our previous work and show that retinoic acid also activates specifically the c-Jun N-terminal kinase (JNK) phosphorylation pathway, which in turn inhibits GFAP expression. Our results suggest the existence of a negative self-regulatory loop in the phosphorylation pathways that regulates GFAP expression. This loop is constitutively repressed by the PI3K pathway. Our results could be relevant for disorders involving sustained GFAP overexpression in precursor cells, such as glioblastoma and Alexander disease.** 

**Key words: GFAP, c-Jun N-terminal kinase, retinoic acid, neural precursor cells.**

Astrocytes are glial cells that play a key role in the function of the central nervous system in normal and pathological conditions. They are normally generated during the perinatal period (Bayer et al., 1993; Skoff et al., 1976), but adult astrogliogenesis can occur in neurodegenerative disorders, central nervous system injuries, and some congenital disorders (Mito and Becker, 1993; Pekny and Nilsson, 2005). Studies on astrogliogenesis very frequently rely on changes in the expression of the glial fibrillary acidic protein (GFAP). GFAP is an intermediate filament typically expressed in astrocytes, but also in other cell types inside and outside the central nervous system (Middeldorp and Hol, 2011). GFAP-null mice are essentially normal, and other intermediate filaments, such as vimentin, can replace most GFAP functions (Menet et al., 2000; Nawashiro et al., 2000; Triolo et al., 2006). However, GFAP-null mice show higher susceptibility to central nervous system insults, suggesting that GFAP is especially important in pathological conditions (Nawashiro et al., 2000; Triolo et al., 2006). Increased GFAP expression levels are associated with more severe reactive gliosis in diverse neuropathological conditions and lower tumorigenicity of subpopulations of glioma cells (Pekny and Nilsson, 2005; Prestegarden et al., 2010). Furthermore, specific mutations in GFAP or its overexpression can cause Alexander disease, a fatal developmental disorder characterized by macrocephaly, leukodystrophy, seizures, and cognitive delays, among other symptoms (Sawaishi, 2009). Therefore, given the relevance of GFAP to pathological conditions, it is important to understand the mechanisms involved in the regulation of its expression.

Current evidence indicates that the signaling pathways involved in the expression of GFAP in normal and pathological conditions are basically the same, all of them involving the phosphorylation of one or more key proteins (Balasingam et al., 1994; Okada et al., 2006; Sriram et al., 2004). The canonical astrogliogenic pathway is the janus kinase/signal transduction and activation of transcription phosphorylation pathway (Balasingam et al., 1994; Barnabe-Heider et al., 2005; Bonni et al., 1997; Nakashima et al., 1999; Rajan and McKay, 1998; Sriram et al., 2004). Most of the other pathways known to participate in the regulation of GFAP expression are connected to this one, including Notch, retinoic acid, tumor growth factor-$\beta$ (TGF-$\beta$), and Wnt pathways (Herrera et al., 2010; Kamakura et al., 2004; Kasai et al., 2005; Nakashima et al., 1999; Rajan et al., 2003). We have previously described a series of changes in the phosphoproteome of neural precursor cells upon their exposure to various astrogliogenic stimuli, such as leukemia inhibitory factor (LIF), fetal bovine serum, or retinoic acid (RA) (Herrera et al., 2009, 2010). In the present work, we examined further the role of phosphorylation pathways in the regulation of GFAP expression by retinoic acid.

## EXPERIMENTAL PROCEDURES

### Materials

The HCN-B27 clone of adult hippocampal precursor cells was isolated in our laboratory as previously described (Schubert et al., 2006). Procedures for cell culture were described elsewhere (Herrera et al., 2009). Retinoic acid was purchased from Sigma (St.

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*Abbreviations:* BMP-2, bone morphogenetic protein-2; DNA-PKcs, DNA-protein kinase catalytic subunit; ERK, extracelular-regulated kinase; GFAP, glial fibrillary acidic protein; GSK-$\beta$, glycogen synthase kinase-$\beta$; JNK, c-Jun N-terminal kinase; LIF, leukemia inhibitory factor; MAPK, mitogen-activated protein kinase; mTOR, mammalian target of rapamycin; PAK1, p21-activated kinase-1; PDK1, phosphoinositide-dependent kinase-1; PI3K, phosphatidylinositol-3-kinase; RA, retinoic acid; TGF-$\beta$, tumor growth factor-$\beta$.

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Louis, MO, USA). Human recombinant bone morphogenetic protein-2 (BMP-2) was obtained from R&D systems (Minneapolis, MN, USA) and LIF from Chemicon (Temecula, CA, USA). Drugs and inhibitors were purchased from Calbiochem (Merck KGaA, Darmstadt, Germany), unless otherwise indicated.

### Immunocytochemistry and Western blots

Procedures for protein extraction and immunoblotting were described previously (Herrera et al., 2009). GFAP antibody (1:2000) was purchased from Chemicon (Temecula). Actin antibody (1:1000) was purchased from Sigma (St. Louis); phospho-PAK1/2/3(Ser141) antibody (1:1000) from Biosource (Invitrogen, Carlsbad, CA, USA); and total c-Jun (1:500) from Santa Cruz Biotechnologies (Santa Cruz, CA, USA). Antibodies against phospho-Akt (Ser473), phospho-FoxO1 (Ser256), phospho-mTOR (Ser2448), phospho-p90RSK (Ser380), phospho-PDK1 (Ser241), phospho-PTEN (Ser380), phospho-S6 ribosomal protein (Ser235/236), phospho-p70 S6 Kinase (Thr389), phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204), phospho-p38 MAPK (Thr180/Tyr182), phospho-SAPK/JNK (Thr183/Tyr185), phosphor-c-Jun (ser63), and the corresponding pan-antibodies (1:1000) were obtained from Cell Signaling Technologies (Danvers, MA, USA).

### Two-dimensional gel electrophoresis

Conditioned media were obtained as previously described (Schubert et al., 2009) and desalted by means of ion-exchange sepharose columns (GE Healthcare, Piscataway, NJ, USA), frozen in dry ice, and lyophilized. Lyophilized proteins were resuspended in 0.5 ml of sample buffer [8 M urea, 4% (w/v) 3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate, 40 mM Tris, 0.2% Bio-Lyte 3–10 ampholytes (Bio-Rad, Hercules, CA, USA), 50 mM dithiothreitol]. Samples were centrifuged at 14000×*g* for 10 min, to remove insoluble proteins. Protein concentration in supernatants was determined by the Lowry assay. Two hundred micrograms of supernatant was loaded onto pH 3–10 isoelectric focusing strips (Bio-Rad) and electrophoresed to 60000 V-h on a Bio-Rad Protean IEF device. The strip was then applied to the top of an 18-cm Bio-Rad precast 12% acrylamide gel and electrophoresed at 25 milliamps per gel until the dye front was at the bottom. Gels were fixed in 50% methanol overnight and stained with silver. Gel spots from 2-D gels were excised and in-gel digested with trypsin and analyzed by LC electrospray ionization MS, as described previously (Schubert et al., 2009).

### Statistical analysis

Results are shown as the mean±standard deviation of at least three independent experiments. Statistical analyses comprised a one-way ANOVA followed by a Student-Newman–Keuls post hoc test. Differences were considered significant when $P<0.05$.

## RESULTS

The HCNB27 clone of adult rat hippocampal precursor cells is a subclone of the HCN cell line (Ray and Gage, 2006) that has been recently isolated and characterized in our laboratory (Herrera et al., 2009, 2010; Schubert et al., 2006). Glial differentiation in these cells is induced to different degrees by retinoic acid and LIF (IL-6 family), and the gliogenic effects of retinoic acid and LIF are enhanced by the BMP-2 (TGF-$\beta$ family) (Herrera et al., 2009, 2010; Nakashima et al., 1999). HCNB27 cells treated with retinoic acid or a combination of retinoic acid, LIF, and BMP showed GFAP expression only after 2 days of incubation with the gliogenic stimuli (Fig. 1A). After this time, GFAP expression increases or remains constant, depending on the gliogenic stimulus, suggesting that the pathways that induce GFAP expression are stably activated over time. Therefore, the phosphorylation state or the expression of proteins was always analyzed after 2 days of incubation with the different compounds, unless otherwise indicated.

An additional marker for differentiation is the complexity and composition of proteins that are released from cells into the extracellular space. We have previously shown that the secretome of neural precursor cells is qualitatively and quantitatively much more complex than the secretome of neurons or astrocytes (Schubert et al., 2009). To know whether the secretome of precursor cells differentiated with retinoic acid and cytokines resembles the secretome of mature astrocytes, 2-D gel proteomic analyses were carried out. Fig. 1B–D shows that there is a sequential decline in the number of extracellular proteins following treatment with retinoic acid, or the more potent combination of retinoic acid, LIF, and BMP. A subset of the proteins in these gels was identified by LC/MS/MS and is shown in Table 1. The levels of secretion of most of the proteins (73.6%) are greatly decreased, including various chaperones and metabolic enzymes. However, there are a few secreted proteins (approximately 15.1%) whose levels are higher after incubation of precursor cells with retinoic acid, LIF, and BMP, including a striking increase in the secretion of procollagen type 3 (Fig. 1B–D, spot nr. 21). This secretome pattern is extremely similar to the pattern of mature astrocytes previously described by our group (Schubert et al., 2009), and is therefore consistent with the differentiation of neural precursor cells into astrocytes.

Retinoic acid and cytokines induce large changes in the phosphoproteome of neural precursor cells that fall primarily within two major classes: phosphoinositol metabolism and mitogen-activated protein kinase (MAPK) pathways (Herrera et al., 2009). Phosphatidylinositol-4,5-bisphosphate-3-kinase (PI3K) is one of the major enzymes involved in phosphoinositol metabolism. The PI3K-specific inhibitor LY294002 and siRNAs against the inositol phosphatase synaptojanin-1 blocked retinoic acid-induced expression of GFAP (Herrera et al., 2009, 2010). Retinoic acid induced the phosphorylation of many proteins of the PI3K pathway, including Akt, mammalian target of rapamycin (mTOR), p70 S6 kinase, S6 ribosomal protein, phosphoinositide-dependent kinase-1 (PDK1), p21-activated kinase-1 (PAK1), and FoxO1 (Fig. 2A). We had previously found that the phosphorylation state of phospholipase C was also changed following treatment with retinoic acid in a PI3K-dependent manner (Herrera et al., 2009), and its inhibition with the specific phospholipase C inhibitor U-73122 (250 nM) strongly enhanced the expression of GFAP (Table 2, Data not shown), suggesting that phospholipase C activity represses GFAP expression. Finally, specific inhibitors for Akt, mTOR, and DNA-protein kinase catalytic subunit (DNA-PKcs) did not have any effect on GFAP expression, even at very high concentrations (Table 2). These results are consistent with our previous observations, suggesting a role for PI3K-regulated inositol metabolism in the regulation of GFAP expression.