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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.

Keywords

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 astroglialogenesis can occur in neurodegenerative disorders, central nervous system injuries, and some congenital disorders (Mito and Becker, 1993; Pekny and Nilsson, 2005). Studies on astroglialogenesis 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.,

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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- β (TGF- β), 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. 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- β 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.

Among the MAPK members, we had previously identified phosphorylation changes in the mitogen-activated protein kinase kinase kinase kinases 4 and 5 (NIK and GCKR, respectively), the Traf2 and NCK interacting kinase (TNIK), and the mitogen-activated protein kinase kinase kinase 6 (ASK2) (Herrera et al., 2009). These kinases are mostly involved in the Stress Activated Protein Kinase/c-Jun N-terminal Kinase (SAPK/JNK) pathway (Fu et al., 1999; Machida et al., 2004; Ramjaun et al., 2001; Takeda et al., 2007). Consistently, retinoic acid induced the phosphorylation of JNK, but not extracellular-regulated kinase (ERK) or p38 MAPK (Fig. 2B). The specific JNK inhibitor SP600125, but not ERK or p38-MAPK inhibitors, potentiated the expression of GFAP induced by retinoic acid (Table 2) (Fig. 2C). Together these results suggest that JNK activation by retinoic acid is a negative feedback loop for the control of GFAP expression.

An extensive set of pharmacological inhibitors against various signaling pathways was unable to modify GFAP expression even at high but subtoxic concentrations, suggesting that

the results observed with the PI3K and JNK inhibitors are specific (Table 2). For example, the glycogen synthase kinase- β (GSK- β) inhibitor lithium chloride (LiCl) did not have any effect on retinoic acid-induced GFAP expression, and retinoic acid did not induce significant changes in the phosphorylation state of GSK- β or β -catenin, two key proteins of this phosphorylation pathway (Data not shown).

Previous results from our laboratory indicated that retinoic acid and cytokines have a synergistic effect on the induction of GFAP expression, and that PI3K is upstream of the entire sequence of events leading to GFAP expression (Herrera et al., 2009, 2010). To situate JNK in this sequence, we analyzed the effects of cytokines and LY294002 on the increase in JNK phosphorylation induced by retinoic acid (Fig. 2D). Cytokines completely blocked the phosphorylation of JNK induced by retinoic acid. On the other hand, LY294002 induced an increase in the phosphorylation of JNK in the presence or absence of retinoic acid and cytokines, suggesting that PI3K is constitutively inhibiting JNK. LY294002 also reversed completely the inhibition of JNK phosphorylation by cytokines. In HCNB27 cells, cytokines also activate PI3K, induce the expression of GFAP, and strongly synergize with retinoic acid in the regulation of GFAP expression. A PI3K-mediated inhibition of JNK phosphorylation by cytokines could explain, at least in part, the synergistic effect of retinoic acid and cytokines on the regulation of GFAP expression (Herrera et al., 2010). Conversely, the inhibitory effect of the PI3K inhibitor LY294002 on retinoic acid- and cytokine-induced GFAP expression could be partially mediated by the activation of JNK.

To know if our results are also relevant to mature astrocytes in the adult brain, we incubated astrocytes with retinoic acid in the presence or absence of the JNK and PI3K inhibitors and tested their effect on GFAP expression. None of the treatments had any effect on GFAP expression (Data not shown). These results indicate that the pathways involved in GFAP expression during astroglial differentiation and in mature astrocytes are different.

Discussion

Our results indicate that retinoic acid-induced GFAP expression has an autoregulatory loop controlled by specific phosphorylation pathways (Fig. 2E). PI3K activation by retinoic acid and cytokines induces the expression of GFAP by mechanisms previously delineated by us and others (Herrera et al., 2009, 2010; Nakashima et al., 1999; Yokogami et al., 2000). However, retinoic acid-induced activation of PI3K also triggers JNK phosphorylation and, possibly, alternative pathways via phosphoinositol metabolism and phospholipase C. These pathways would act as part of a negative feedback loop for the control of GFAP expression, preventing precursor cells from overexpressing GFAP during their differentiation into astrocytes. Most of the results from our laboratory are consistent with two cell populations, one more sensitive to retinoic acid and another to cytokines, that express more GFAP in the presence of an extra gliogenic stimulus and would therefore share the same intracellular pathway. Although this is our working hypothesis, we cannot rule out the recruitment of more cell populations toward an astroglial fate, which would add several levels of complexity to the pathway we propose in the present article.

GFAP overexpression causes the generation of Rosenthal fibers, the main histopathological hallmark of Alexander disease and other neuropathologies (Middeldorp and Hol, 2011; Sawaishi, 2009). On the other hand, GFAP depletion is easily compensated for by other intermediate filaments and has no overt phenotype (Menet et al., 2000; Nawashiro et al., 2000; Triolo et al., 2006). As a consequence, the specific blockage of GFAP expression would be an ideal therapy against GFAP-related disorders. The fact that the expression of GFAP is not affected by retinoic acid or the JNK and PI3K inhibitors in primary cultures of astrocytes strongly suggests that the pathways regulating GFAP expression are different during development and in the adult brain. We have previously shown that retinoic acid and cytokines have a synergistic effect on GFAP expression and the number of GFAP-positive cells, suggesting that their combination recruits new cell populations toward an astroglial fate (Herrera et al., 2010). Further understanding of the mechanisms that control GFAP expression could contribute to the design of new therapies for these disorders.

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References

- Balasingam V, Tejada-Berges T, Wright E, Bouckova R, Yong VW. Reactive astrogliosis in the neonatal mouse brain and its modulation by cytokines. *J Neurosci.* 1994; 14:846–856. [PubMed: 8301364]
- Barnabe-Heider F, Wasylnka JA, Fernandes KJ, Porsche C, Sendtner M, Kaplan DR, Miller FD. Evidence that embryonic neurons regulate the onset of cortical gliogenesis via cardiotrophin-1. *Neuron.* 2005; 48:253–265. [PubMed: 16242406]
- Bayer SA, Altman J, Russo RJ, Zhang X. Timetables of neurogenesis in the human brain based on experimentally determined patterns in the rat. *Neurotoxicology.* 1993; 14:83–144. [PubMed: 8361683]
- Bonni A, Sun Y, Nadal-Vicens M, Bhatt A, Frank DA, Rozovsky I, Stahl N, Yancopoulos GD, Greenberg ME. Regulation of gliogenesis in the central nervous system by the JAK-STAT signaling pathway. *Science.* 1997; 278:477–483. [PubMed: 9334309]
- Fu CA, Shen M, Huang BC, Lasaga J, Payan DG, Luo Y. TNIK, a novel member of the germinal center kinase family that activates the c-Jun N-terminal kinase pathway and regulates the cytoskeleton. *J Biol Chem.* 1999; 274:30729–30737. [PubMed: 10521462]
- Herrera F, Chen Q, Fischer WH, Maher P, Schubert DR. Synaptojanin-1 plays a key role in astrogliogenesis: possible relevance for Down's syndrome. *Cell Death Differ.* 2009; 16:910–920. [PubMed: 19282871]
- Herrera F, Chen Q, Schubert D. Synergistic effect of retinoic acid and cytokines on the regulation of glial fibrillary acidic protein expression. *J Biol Chem.* 2010; 285:38915–38922. [PubMed: 20876578]
- Kamakura S, Oishi K, Yoshimatsu T, Nakafuku M, Masuyama N, Gotoh Y. Hes binding to STAT3 mediates crosstalk between Notch and JAK-STAT signalling. *Nat Cell Biol.* 2004; 6:547–554. [PubMed: 15156153]
- Kasai M, Satoh K, Akiyama T. Wnt signaling regulates the sequential onset of neurogenesis and gliogenesis via induction of BMPs. *Genes Cells.* 2005; 10:777–783. [PubMed: 16098141]
- Machida N, Umikawa M, Takei K, Sakima N, Myagmar BE, Taira K, Uezato H, Ogawa Y, Kariya K. Mitogen-activated protein kinase kinase kinase 4 as a putative effector of Rap2 to activate the c-Jun N-terminal kinase. *J Biol Chem.* 2004; 279:15711–15714. [PubMed: 14966141]

- Menet V, Gimenez YR, Sandillon F, Privat A. GFAP null astrocytes are a favorable substrate for neuronal survival and neurite growth. *Glia*. 2000; 31:267–272. [PubMed: 10941153]
- Middeldorp J, Hol EM. GFAP in health and disease. *Prog Neurobiol*. 2011; 93:421–443. [PubMed: 21219963]
- Mito T, Becker LE. Developmental changes of S-100 protein and glial fibrillary acidic protein in the brain in Down syndrome. *Exp Neurol*. 1993; 120:170–176. [PubMed: 8491278]
- Nakashima K, Yanagisawa M, Arakawa H, Kimura N, Hisatsune T, Kawabata M, Miyazono K, Taga T. Synergistic signaling in fetal brain by STAT3-Smad1 complex bridged by p300. *Science*. 1999; 284:479–482. [PubMed: 10205054]
- Nawashiro H, Brenner M, Fukui S, Shima K, Hallenbeck JM. High susceptibility to cerebral ischemia in GFAP-null mice. *J Cereb Blood Flow Metab*. 2000; 20:1040–1044. [PubMed: 10908037]
- Okada S, Nakamura M, Katoh H, Miyao T, Shimazaki T, Ishii K, Yamane J, Yoshimura A, Iwamoto Y, Toyama Y, Okano H. Conditional ablation of Stat3 or Socs3 discloses a dual role for reactive astrocytes after spinal cord injury. *Nat Med*. 2006; 12:829–834. [PubMed: 16783372]
- Pekny M, Nilsson M. Astrocyte activation and reactive gliosis. *Glia*. 2005; 50:427–434. [PubMed: 15846805]
- Prestegarden L, Svendsen A, Wang J, Sleire L, Skafnesmo KO, Bjerkvig R, Yan T, Askland L, Persson A, Sakariassen PO, Enger PO. Glioma cell populations grouped by different cell type markers drive brain tumor growth. *Cancer Res*. 2010; 70:4274–4279. [PubMed: 20460538]
- Rajan P, McKay RD. Multiple routes to astrocytic differentiation in the CNS. *J Neurosci*. 1998; 18:3620–3629. [PubMed: 9570793]
- Rajan P, Panchision DM, Newell LF, McKay RD. BMPs signal alternately through a SMAD or FRAP-STAT pathway to regulate fate choice in CNS stem cells. *J Cell Biol*. 2003; 161:911–921. [PubMed: 12796477]
- Ramjaun AR, Angers A, Legendre-Guillemain V, Tong XK, McPherson PS. Endophilin regulates JNK activation through its interaction with the germinal center kinase-like kinase. *J Biol Chem*. 2001; 276:28913–28919. [PubMed: 11384986]
- Ray J, Gage FH. Differential properties of adult rat and mouse brain-derived neural stem/progenitor cells. *Mol Cell Neurosci*. 2006; 31:560–573. [PubMed: 16426857]
- Sawaishi Y. Review of Alexander disease: beyond the classical concept of leukodystrophy. *Brain Dev*. 2009; 31:493–498. [PubMed: 19386454]
- Schubert D, Herrera F, Cumming R, Read J, Low W, Maher P, Fischer WH. Neural cells secrete a unique repertoire of proteins. *J Neurochem*. 2009; 109:427–435. [PubMed: 19200335]
- Schubert D, Klar A, Park M, Dargusch R, Fischer WH. F-spondin promotes nerve precursor differentiation. *J Neurochem*. 2006; 96:444–453. [PubMed: 16300627]
- Skoff RP, Price DL, Stocks A. Electron microscopic autoradiographic studies of gliogenesis in rat optic nerve. II. Time of origin. *J Comp Neurol*. 1976; 169:313–334. [PubMed: 972202]
- Sriram K, Benkovic SA, Hebert MA, Miller DB, O'Callaghan JP. Induction of gp130-related cytokines and activation of JAK2/STAT3 pathway in astrocytes precedes up-regulation of glial fibrillary acidic protein in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine model of neurodegeneration: key signaling pathway for astroglialosis in vivo? *J Biol Chem*. 2004; 279:19936–19947. [PubMed: 14996842]
- Takeda K, Shimozone R, Noguchi T, Umeda T, Morimoto Y, Naguro I, Tobiume K, Saitoh M, Matsuzawa A, Ichijo H. Apoptosis signal-regulating kinase (ASK) 2 functions as a mitogen-activated protein kinase kinase in a heteromeric complex with ASK1. *J Biol Chem*. 2007; 282:7522–7531. [PubMed: 17210579]
- Triolo D, Dina G, Lorenzetti I, Malaguti M, Morana P, Del CU, Comi G, Messing A, Quattrini A, Previtali SC. Loss of glial fibrillary acidic protein (GFAP) impairs Schwann cell proliferation and delays nerve regeneration after damage. *J Cell Sci*. 2006; 119:3981–3993. [PubMed: 16988027]
- Yokogami K, Wakisaka S, Avruch J, Reeves SA. Serine phosphorylation and maximal activation of STAT3 during CNTF signaling is mediated by the rapamycin target mTOR. *Curr Biol*. 2000; 10:47–50. [PubMed: 10660304]

Abbreviations

BMP-2	bone morphogenetic protein-2
DNA-PKcs	DNA-protein kinase catalytic subunit
ERK	extracellular-regulated kinase
GFAP	glial fibrillary acidic protein
GSK-β	glycogen synthase kinase- β
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-β	tumor growth factor- β

Fig. 1.

Retinoic acid and cytokines induce glial differentiation and a decrease in the number and complexity of secreted proteins. (A) Retinoic acid (RA or R, 1 μ M) induces an increase in the expression of GFAP after 2 days of incubation. GFAP expression is greatly enhanced when retinoic acid is added in combination with the cytokines bone morphogenetic protein-2 (BMP, 50 ng/ml) and leukemia inhibitory factor (LIF, 50 ng/ml). The expression of GFAP peaks after 4 days of incubation with retinoic acid alone. On the other hand, the combination of retinoic acid and cytokines initially induces a much higher increase in the expression of GFAP, which remains constant for at least 2 more days. *, significant versus control (CON, C); #, significant versus retinoic acid (RA, R), $P < 0.05$. (B–D) Silver-stained 2-D gels of proteins extracted from conditioned media after 2 days of incubation with the different gliogenic stimuli. (B) Control; (C) Retinoic acid; (D) Retinoic acid, BMP, and LIF. In the horizontal axis, proteins are separated according to their isoelectric point and in the vertical axis according to their molecular weight (kDa). Fifty-three proteins were identified by LC/MS/MS, numbered, and shown in Table 1. Red highlighted numbers indicate proteins whose secretion is decreased upon exposure of cells to gliogenic stimuli. Green highlighted numbers indicate astrocyte-specific secreted proteins. Black numbers indicate proteins that do not experience substantial changes during astroglial differentiation. For interpretation of

the references to color in this figure legend, the reader is referred to the Web version of this article.

Fig. 2.

Phosphorylation pathways involved in retinoic acid-induced regulation of GFAP expression. All the immunoblots show results obtained after 2 days of incubation with the different compounds. (A, B) Incubation of HCNB27 precursor cells with retinoic acid (RA, R, 1 μ M) for 2 days specifically induces the phosphorylation of key residues of members of the PI3K (A) and the JNK pathways (B). *, significant versus control group (CON, C), $P < 0.05$. (C) The specific JNK inhibitor SP600125 (JNK inh, J, 10 μ M) potentiates retinoic acid-induced expression of GFAP and effectively blocks JNK and c-Jun phosphorylation. *, significant versus control group (CON, C); #, significant versus corresponding retinoic acid-treated group, $P < 0.05$. (D) Retinoic acid (RA, R, 1 μ M) induces an increase in the phosphorylation of JNK. The cytokines BMP-2 (BMP, B, 50 ng/ml) and LIF (L, 50 ng/ml) inhibit retinoic acid-induced phosphorylation of JNK. The PI3K-specific inhibitor LY294002 (10 μ M) enhances JNK phosphorylation in the presence or absence of retinoic acid and cytokines, blocking completely the inhibitory effect of cytokines on JNK phosphorylation. *, significant versus control group (CON); #, significant versus corresponding DMSO-treated groups, $P < 0.05$. (E) Summary of results. Retinoic acid activates both PI3K and JNK, which have opposing effects on GFAP expression. PI3K activates GFAP expression, whereas JNK inhibits it. Cytokines activate PI3K, and PI3K constitutively inhibits JNK. This could explain at least part of the induction of GFAP expression by PI3K activation and the synergy between cytokines and retinoic acid. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

Table 1

Table of secreted proteins. The secretion of a few proteins is increased during glial differentiation, but the levels of most secreted proteins show a large decrease. Each protein is assigned an accession number (GI number) and theoretical molecular weight. The number of peptides needed for the identification of secreted proteins and the probability-based Mowse score determined by the Mascot search algorithm are listed. Scores >40 are considered significant. Each protein spot in this table has been identified by LC/MS/MS at least three times in the same position on the gels from three independent experiments. Spot numbers correspond to the labeled spots in the 2-D gels (Fig. 2A–C)

Spot number	Protein name	GI number	MW (kDa)	Score	Peptides	CON	RA	BLR
21	Procollagen type 3 (fragment)	57916	62.2	317	33	(-)	+	+
47	Macrophage migration inhibitory factor beta chain	5542287	12.3	210	2	-	-	+
48	Tumor metastatic protein NM 23	387496	18.0	128	8	-	-	+
49	Follistatin-related protein 7	2498391	34.5	90	8	-	-	+
50	Creatine kinase	10946574	42.6	409	4	-	-	+
51	Ser/Cys protease inhibitor F1	29293811	46.4	596	23	-	-	+
52	Dihydropyrimidinase 3 (fragment)	21666559	738.5	162	5	-	-	+
53	Procollagen C-proteinase enhancer protein	9506953	50.2	496	31	-	-	+
1	Fatty acid-binding protein 5	6754450	15.1	101	3	+	(-)	(-)
2	Expressed in nonmetastatic cell protein 1	56800494	17.2	197	20	+	(-)	-
4	Glutathione S-transferase-p12	25453412	23.4	434	27	+	(-)	-
5	Triosephosphate isomerase 1	54855	26.7	162	5	+	(-)	-
6	Glutathione S-transferase YtyfA	576133	23.5	220	18	+	(-)	(-)
7	Proteasome alpha type 5	6679497	25.9	68	2	+	(-)	-
8	Triosephosphate isomerase	54855	26.7	162	5	+	(-)	-
9	Pyroline-5-carboxylate reductase	13384800	26.2	56	1	+	+	-
11	Tumor metastatic protein NM 23	387496	18.7	144	3	+	+	-
12	Tumor metastatic protein NM 23	387496	18.7	121	4	+	+	-
13	Tumor metastatic protein NM 23	387496	18.7	78	3	+	-	-
14	Proteasome beta 3	83944082	22.9	116	2	+	(-)	-
15	Endoplasmic reticulum p29	19526463	28.8	171	7	+	+	-
16	Phosphatidyl ethanolamine-binding protein	92565972	20.8	144	5	+	-	-
17	Tyrosine hydroxylase	31981925	29.2	314	17	+	-	-
18	Proteasome alpha 3	3914438	28.4	264	22	+	-	-

Spot number	Protein name	GI number	MW (kDa)	Score	Peptides	CON	RA	BLR
19	Inositol phosphatase	3914098	30.4	139	8	+	-	-
20	Heat shock protein (HSP27)	547679	22.9	204	10	+	(-)	-
22	Phosphatidylinositol transfer protein alpha	6679337	31.9	155	4	+	-	-
23	Malic dehydrogenase	14152	64.9	78	5	+	+	-
24	Lactate dehydrogenase chain M	65923	36.5	60	1	+	-	-
25	Aspartate aminotransferase	871422	46.2	154	11	+	+	-
26	Aldolase 3	14198249	39.3	145	4	+	(-)	-
27	Alpha fetoprotein	191765	47.2	81	2	+	+	-
28	Aspartate aminotransferase	811422	46.2	154	11	+	-	-
29	Dihydropyridinase-2	40254595	62.2	446	25	+	+	-
30	Alpha fetoprotein	191765	47.2	84	14	+	-	-
31	Transaldolase	33859640	33.4	130	5	+	+	-
32	LOC433182 protein	53734652	50.2	800	128	+	+	-
33	GDP dissociation inhibitor 1	40254781	50.5	631	63	+	+	-
34	Gamma-actin	4885049	41.0	152	7	+	(-)	-
35	Beta actin	49868	39.2	295	19	+	(-)	-
36	Tubulin alpha 3	90217	50.1	597	77	+	(-)	-
37	Prolyl-4-hydroxylase beta	54777	57.1	201	9	+	(-)	-
38	Heat shock protein 70 (HSP70)	1661134	70.9	410	25	+	-	-
39	Transketolase	12018252	71.1	616	76	+	(-)	-
40	Thioredoxin reductase 1	5410338	54.2	324	47	+	(-)	-
41	M2 pyruvate kinase	1675994	57.7	509	16	+	(-)	-
43	Heat shock protein 70 (HSP70)	1661134	70.9	655	33	+	-	-
44	F-spondin	50510627	82.0	248	9	+	-	-
45	Heat shock protein 90 (HSP 90)	40556608	83.2	1045	33	+	(-)	-
46	Elongation factor 2	600,159	95.2	329	18	+	(-)	-
3	Cyclophilin C	1000036	19.9	51	2	+	-	+
10	Glutathione S-transferase Yfyf B	2624496	23.4	50	1	+	-	+
42	Tuc 46	21666559	73.9	162	3	+	+	+
54	Endoplasmic reticulum p29	19526463	28.8	171	7	+	+	+

-, Protein absent.

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(-), Protein present, but at levels lower than CON.
+, Protein present.
CON, control; RA, retinoic acid treated; BLR, treated with BMP, LIF, and retinoic acid.

Table 2
Effect of inhibitors against multiple intracellular signaling pathways on retinoic acid-induced GFAP expression

Inhibitor name (concentration)	Target	Effect on RA-induced GFAP expression
SP600125 (10 μ M)	JNK	+
U-73122 (250 nM)	Phospholipase C	+
Akt inhibitor I (10 μ M)	Akt	None
Aphidicolin (10 μ M)	G1/S Cell cycle transition	None
DNA-PK inhibitor II (10 μ M)	DNA-PKcs	None
FH535 (100 nM)	β -catenin	None
Go6983 (5 μ M)	Protein kinase C (all isoforms)	None
KN-93 (2.5 μ M)	Calmodulin-dependent kinase II	None
LiCl (20 mM)	GSK β	None
Olomoucine (50 μ M)	Cyclin-dependent kinases (CDKs)	None
PD98059 (50 μ M)	MEK (upstream ERK)	None
Rapamycin (1 μ M)	mTOR	None
SB203580 (10 μ M)	P38-MAPK	None
SU6656 (10 μ M)	Src kinase	None
W-7 (10 μ M)	Calmodulin	None

+, Potentiation; None, no effect.