Glial fibrillary acidic protein (GFAP), is the main astroglial marker during astrogliogenesis, but it is also expressed in other cell types, including neural stem cells and old neurons. Activation of the JAK/STAT pathway by the IL-6 family of cytokines is the canonical pathway regulating GFAP expression, while retinoic acid is thought to be the only inducer of GFAP to operate independently of this pathway. Here we show that retinoic acid receptor alpha (RARα) not only links retinoic acid signaling to the canonical cytokine-stimulated pathway leading to GFAP expression, but also plays a key role in the synergistic actions of retinoic acid and cytokines on this pathway. Cytokines both potentiate RARα expression and enhance its binding to DNA and to the Stat3/p300/CBP/Smad transcriptional complex, the cornerstone of the canonical pathway. PI3K is upstream all the key events leading to the expression of GFAP. Our results give new insights about the role of retinoic acid signaling in GFAP expression.

The intermediate filament protein glial fibrillary acidic protein (GFAP) is considered a cell type-specific marker for astrocytes. However, GFAP is expressed in some nerve cell populations, including hippocampal neurons of old individuals (1). It is also expressed in some precursor populations of nerve as well as in cells that can give rise to both nerve and glia (2-4). Finally, the expression levels of GFAP within individual cells is extremely variable, and higher expression levels may limit the tumorigenicity of subpopulations of cells within glioblastomas (5,6). Because of the wide range of cellular phenotypes associated with GFAP, it is important to understand the molecular mechanisms that regulate its expression.

The GFAP is regulated, in part by the secretion of factors into the extracellular space (7). The canonical pathway for GFAP expression in astrocytes is triggered by the binding of cytokines from the interleukin-6 (IL-6) family to their receptors. These receptors subsequently activate the JAK/STAT intracellular pathway, leading to the expression of GFAP in astrocytes (7-9). Most of the other pathways known to participate in GFAP expression are connected at some point to this canonical pathway (10,11). For example, some members of the tumor growth factor-beta (TGF-β) superfamily of cytokines have little or no effect on GFAP synthesis by themselves, but they strongly potentiate GFAP induced by the IL-6 family of cytokines. They do so by triggering the phosphorylation, activation and heterodimerization of Smad proteins, which then form a more potent transcriptional complex with p300/CBP and Stat3. As a consequence, the expression of GFAP is greatly enhanced (9), (12-14).

All-trans retinoic acid (Retinoic acid, RA), generally considered to be a neurogenic agent, but is, in fact, a potent inducer of GFAP (14-18). Studies with pharmacological agonists for the different retinoic acid receptors indicate that retinoic acid-induced GFAP expression is mediated by the activation of retinoic acid receptor α (RARα) and, to a lesser extent, retinoic acid receptor γ (RAR γ) (16,17). Other than a potential role for these receptors, little is known about the mechanisms involved in retinoic acid-induced GFAP synthesis.

The relationship between retinoic acid and cytokine signaling pathways depends strongly upon the biological context. For example, retinoic acid inhibits GFAP-associated astrocyte differentiation induced by ciliary neurotrophic...
factor (CNTF), an IL-6 family cytokine, in rat neural precursor cells isolated from embryonic day 13 brains, but potentiates it in precursor cells from embryonic day 17 brains (16). Very recently, Asano and co-workers also showed that retinoic acid and the IL-6 family of cytokines have a synergistic effect on astrogenesis in mouse neural precursor cells from embryonic day 14.5 (19). Outside the CNS, the combination of retinoic acid with cytokines enhances some biological effects (20,21) and counteracts others (22). Thus, while it is clear that there is some kind of interaction between retinoic acid and cytokine signaling pathways, the precise nature of this interaction is unclear.

In the work outlined below, we examined the interaction between retinoic acid and cytokine signaling pathways in the control of GFAP expression. Our results provide new insights into the pathways underlying the expression of GFAP, and into the role of cytokine and retinoic acid signaling in the CNS.

**EXPERIMENTAL PROCEDURES**

*Matters* - The HCN-B27 clone of adult hippocampal precursor cells was isolated in our laboratory as previously described (23) as a subclone of the rat adult hippocampal precursor cell line (HCA) (15). Procedures for cell culture were described elsewhere (14). Briefly, cells were maintained in Neurobasal medium plus B27 supplement (w/o antioxidants, Gibco) and seeded in DMEM/Ham’s F12 1:1 medium plus N2 supplement (GIBCO) for all experiments. Retinoic acid was purchased from Sigma (St. Louis, MO, USA) and retinoic acid receptor agonists and antagonists from Biomol International LP (Enzo Life Sciences International Inc., Plymouth Meeting, PA, USA). LY294002 was purchased from Invivogen (Carlsbad, CA, USA), and luciferase assays were carried out by means of the Dual-light combined reporter gene assay system (Applied Biosystems Inc., Foster City, CA, USA). In all cases, manufacturer’s instructions were followed.

Nuclear protein extracts were prepared with a kit from Epigentek (Brooklyn, NY, USA). The binding of RARα to the half retinoic acid response element identified in the rat GFAP promoter was determined by the EpiQuik™ General Protein-DNA Binding Assay Kit (Fluorometric) from Epigentek (Brooklyn, NY, USA). This kit is designed for measuring the binding activity of transcription factors to DNA in nuclear extracts. Briefly, the RARα present in the nuclear extracts of precursor cells is captured by a specific antibody onto the assay microwells. A double-strand oligonucleotide containing the half retinoic acid response element previously identified is added into the microwell and bound to the active RARα in the binding assay buffer. The bound oligonucleotide is then detected fluorometrically. Manufacturer’s instructions were followed for both the nuclear extraction and the protein-DNA binding assay.

*Western blot immunoprecipitation and siRNA* – Procedures for protein extraction and western blotting were described previously (14). For western blots, antibodies against the following proteins were used at the indicated dilutions, GFAP (1,2000) from Chemicon (Temecula, CA, USA); Actin (1,1000, Sigma, St. Louis, MO, USA); GAPDH (1,10,000, Labfrontier, Republic of South Korea); p300/CBP (1,500, Santa Cruz Biotechnologies, Santa Cruz, CA, USA); phospho-Stat3(Ser727), phospho-Stat3 (Tyr705), Stat3, phospho-Smad1(Ser463/465)/ Smad5(Ser463/465)/ Smad8 (Ser426/428), Smad1 and RARα (1,1000, Cell Signaling Technologies, Danvers, MA, USA).

For immunoprecipitations, antibodies against the following proteins were used at the indicated dilutions, p300/CBP and RARα (1,20, Santa Cruz Biotechnologies, Santa Cruz, CA, USA); and Stat3 and Smad1 (1,100 and 1,200, Cell Signaling Technologies, Danvers, MA, USA). The protocol suggested by Cell Signaling Technologies for immunoprecipitation was followed in all cases. Briefly, 400 µg of protein in 100 µl of lysis buffer were incubated with the selected antibodies overnight at 4°C with rotation. The next day, the
samples were incubated with protein A (for rabbit antibodies) or protein G (for mouse antibodies) agarose for another 3 hours at 4°C with rotation. Samples were washed 5 times and resuspended in 20 µl of SDS 3X buffer, boiled for 3 minutes and spun down. The supernatants were run on SDS-PAGE gels, the proteins transferred to PVDF membranes and blotted with the antibodies using general western blot methods, as described previously (14).

siRNA against p300/CBP was purchased from Santa Cruz Biotechnologies (Santa Cruz, CA, USA). It was introduced into the cells by transfection with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA).

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

RARα is involved in retinoic acid-induced GFAP expression - GFAP expression in the HCNB27 clone of adult rat neural precursor cells can be induced to different degrees by retinoic acid, leukemia inhibitory factor (LIF, IL-6 family) and fetal bovine serum (FBS) (Fig. 1, A-C). Although there is not a significant increase in GFAP expression when cells are incubated with bone morphogenetic protein-2 (BMP-2) alone, BMP-2 strongly potentiates the effect of LIF (Fig. 1, A-B), consistent with previous observations by us and others (25), (13,14). The addition of FBS to this combination of cytokines or to retinoic acid alone leads to an additive effect on GFAP levels; i.e. the final effect is more or less equal to the sum of the specific effects of serum and the compounds. In contrast, when retinoic acid is added to the cultures in combination with the cytokines, the final effect is much greater than the sum of the effects of retinoic acid and cytokines, indicating that they synergize (Fig. 1, A-C). Further addition of FBS does not increase the effect of cytokines plus retinoic acid. Importantly, these effects on GFAP expression are seen at physiological concentrations of retinoic acid (nanomolar range) (Fig. 1D), indicating that our observations are not the result of using supraphysiological concentrations of retinoic acid.

Previous reports indicated that retinoic acid-induced GFAP expression in vitro was mediated by RARα and, to a lesser extent, by RARγ (16, 17). In HCNB27 cells, the RARα specific agonist AM-580 induced GFAP, but to a lesser extent than the pan-RAR agonists retinoic acid and a retinoid acid (TTNPB) (Fig. 1E), indicating that RARα activation is sufficient for its expression but that other RARs probably contribute to the maximal response. On the other hand, Ro-415253 (Ro, 250 nM), a specific RARγ antagonist, completely blocked retinoic acid-induced GFAP expression (Fig. 1E), further supporting the idea that RARα activation is necessary (16,17).

RARα is also involved in cytokine-induced GFAP expression - In order to determine if the synergism between retinoic acid and cytokines was at the transcriptional level, HCNB27 cells transfected with plasmids encoding luciferase under the control of the full length rat GFAP promoter were incubated with combinations of retinoic acid and either BMP-2 or LIF, and the luciferase activity was determined (Fig. 2, A-B) (24). The combination of either LIF or BMP-2 with RA had a synergistic effect on the activity of the promoter (Fig. 2B), indicating that both cytokines contribute to the synergism and that the synergy occurs at the transcriptional level.

In order to identify the response element responsible for retinoic acid-induced expression of GFAP, the expression of luciferase was placed under the control of serial deletions of the rat GFAP promoter (Fig. 2A) (24), and HCNB27 cells were exposed to retinoic acid (Fig. 2C). There was a striking decrease in retinoic acid-induced luciferase activity upon deletion of the terminal fragment from -1876 to -1546, indicating that this region contains a response element for RARα. The classic consensus retinoic acid response element consists of a direct repeat of the sequence AGGTCA with a 2 to 5 pair base spacer. We searched the rat GFAP promoter for classical retinoic acid response elements by means of the Tsitescan free software (http://www.ifti.org/cgi-bin/ifti/Tsitescan.pl), but we were unable to find any. The closest sequence we found was a PPARγ/RORα response element situated between positions -1843 and -1837 (AGGTCA), which is half of the consensus retinoic acid response element. The specific deletion of this site from the GFAP promoter blocked retinoic acid-induced luciferase activity, strongly indicating that this sequence is acting as the response element for retinoic acid signaling in the GFAP promoter (Fig. 2, A, D and E). Interestingly, we also found this half response element, but not the classical response element, in the mouse and human GFAP promoters (positions -192 and -627, -1118 and -1298, respectively), indicating that it could also be relevant for retinoic acid-induced glial differentiation in mouse and human cells. However, we cannot exclude the presence of classical RAREs further than 2 Kb from the start of the GFAP gene, the promoter length we
analyzed. A recent report from Asano and co-workers described a full RARE at 2.5 Kb from the start of the mouse GFAP gene (19). This RARE exerts an inhibitory effect on LIF-induced GFAP expression. While its deletion or mutation has no visible effect on the expression of GFAP induced by retinoic or the combination of retinoic acid and LIF, it potentiates GFAP expression induced by LIF alone. Therefore, classical RAREs may have a role on GFAP expression different from the half response element we identified.

The deletion of the half retinoic acid response element also partially blocked the synergistic effect of retinoic acid and cytokines and, most surprisingly, the transcriptional activity induced by LIF alone (Fig. 2, D-E). Furthermore, consistent with a role for RARα in LIF-induced GFAP expression, the RARα specific antagonist Ro-415253 (Ro, 250 nM) partially prevented GFAP expression induced by either LIF alone, the combination of retinoic acid and cytokines, or BMP plus LIF (Figs. 2F and 4A).

Cytokines potentiate the retinoic acid pathway toward GFAP – There are a few key events in the retinoic acid and cytokine GFAP expression pathways that have been extensively confirmed. In the case of the cytokine pathway, these key events are the phosphorylation and subsequent activation of Smad and Stat proteins and their binding to p300/CBP (25), (9). In the case of the retinoic acid pathway, it is the only activation of RARα (Fig. 1E) (16,17). Based on the transcriptional data, we hypothesized that the crosstalk between retinoic acid and the cytokine pathways that leads to the observed synergism involved one or more of these transcription factors. To determine how the transcription factors regulate this synergism, we first examined the phosphorylation state of key activation residues in Smad1/5/8 and Stat3 and the expression of RARα after incubation with the different combinations of cytokines (Fig. 3, A-D). LIF induced the phosphorylation of Stat3 at Tyr705 and Ser727 and BMP-2 the phosphorylation of Smad1/5/8, as expected, but retinoic acid did not potentiate any of these events. Interestingly, retinoic acid induced a decrease in RARα expression that could represent a negative feedback, and a decrease in Smad1 expression that is prevented by BMP-2 but not by LIF (Figs. 3, A, B and D). On the other hand, both BMP-2 and LIF promoted RARα expression and reduced significantly the loss of RARα induced by retinoic acid (Fig. 3, A and B). Cytokines also potentiated the binding of RARα to the half response element that we identified (Fig. 3E) and, furthermore, they potentiated the transcriptional activity of retinoic acid receptors when luciferase expression was under the control of the classical DR-5 retinoic acid response element (Fig. 3F). Together, these results indicate that cytokines potentiate the retinoic acid GFAP pathway by up-regulating RARα expression and activity.

PI3K orchestrates the main events leading to GFAP expression - We have previously shown that PI3K plays a key role in both retinoic acid- and cytokine-induced astrogliogenesis (14). In order to know if it is also involved in the synergism between retinoic acid and cytokines, cells were incubated with the cytokine and/or retinoic acid with or without the specific PI3K inhibitor LY294002 (LY, 10 μM) or the specific RARα inhibitor Ro-415253 (Ro, 250 nM). While the RARα inhibitor had a partial antagonistic effect on GFAP expression, LY294002 completely blocked the GFAP accumulation in response to both the combination of cytokines and the cytokines plus retinoic acid (Fig. 4A). Furthermore, the PI3K inhibitor completely blocked the up-regulation of RARα expression and the increase in the phosphorylation of Stat3 and Smad induced by LIF and BMP, respectively (Fig. 4, B-F). In contrast, the RARα inhibitor did not block these events (Fig. 4B, Supplementary Fig. 1).

The formation of the Stat3/p300/CBP/Smad transcriptional complex is essential for the synergism between LIF and BMP-2 (25) and p300/CBP is critical for retinoic acid signaling in other experimental models (26,27). We observed that knocking down the expression of p300/CBP partially blocked retinoic acid- and cytokine-induced GFAP expression (Fig. 5A), supporting a role for p300/CBP in both pathways. Interestingly, the decrease in p300/CBP expression also leads to down-regulation of the expression of RARα (Fig. 5A). Based on these data, we hypothesized that RARα can form a complex with p300/CBP, Stat3 and Smad proteins during astrogliogenesis. As expected, the binding of Stat3 and Smad1/5/8 proteins to p300/CBP was induced by LIF and BMP-2, respectively (Fig. 5B). Both cytokines induced the binding of RARα to p300/CBP in the presence or absence of retinoic acid, and this effect was much greater when LIF was combined with retinoic acid. While the specific RARα inhibitor Ro-415253 only reduced the binding of RARα to p300/CBP (Fig. 5C), the PI3K specific inhibitor LY294002 reduced the binding of Smad, Stat3 and RARα to p300/CBP (Fig. 5D). Thus, our results strongly indicate that PI3K is upstream of all the key events leading to GFAP expression in both nerve-glial precursor populations and astrocytes induced by either retinoic acid or cytokines, and that it also regulates the crosstalk and synergy between these two pathways.
DISCUSSION

We and others have demonstrated that RARα is essential for retinoic acid-induced GFAP expression (Fig. 1E) (16,17). However, the observation that the specific RARα antagonist Ro-415253 reduces cytokine-induced GFAP expression as well suggests that cytokines also require RARα for the full induction of this protein. Here we show that cytokines potentiate the expression of RARα, its binding to DNA and to the Stat3/p300/CBP/Smad complex, as well as the transcriptional activity induced by retinoic acid (Figs. 3A-B, 3D-E and 5A-C). However, cytokines do not increase classical DR-5 RARE-mediated transcriptional activity by themselves (Fig. 3F). The mechanism of action of Ro-415253 is not well understood, but it is known that it does not prevent RARs from binding to DNA or to other RARs and RXRs. The existing evidence suggests that it causes a change in the final conformation of the RARα protein that makes the receptor inactive (28). We observed that this antagonist reduced the cytokine-stimulated binding of RARα to p300/CBP (Fig. 5B). This may be explained by a change in conformation, explaining the effect of Ro-415253 on RARα activity and on cytokine-induced GFAP expression. Altogether, our results strongly suggest that RARα plays a central role in the regulation of GFAP expression and that it acts as an important cofactor of the Stat3/p300/CBP/Smad complex.

Our results are supported and complemented by reports showing synergism between retinoic acid and the IL-6 family of cytokines in the induction of GFAP expression in precursor cells (16), (19). Retinoic acid has been proposed to potentiate LIF-induced astrogliogenesis by means of an epigenetic mechanism involving histone acetylation (19). p300/CBP has histone acetyltransferase activity, which is regulated by its binding to various transcription factors, such as the Stat, Smad or RAR families (12), (26,27). Here, we showed that RARα binds to p300/CBP in the context of GFAP regulation. Thus, RARα could potentiate p300/CBP acetyltransferase activity in collaboration with Stat and Smad proteins, leading to acetylation of histones near the GFAP promoter, further exposing the promoter to Stat3 and Smad transcription factors and, ultimately, to an enhancement of GFAP expression.

Based on these data, we present a revised version of the canonical pathway for the regulation of GFAP expression during development. Precursor cells and the promoters for genes such as GFAP that are expressed in astrocytes and genes from the canonical astrocyte JAK/STAT pathway must be derepressed and demethylated (Fig. 6, step 1) (29). In the presence of cytokines from the IL-6 family, the JAK/STAT pathway is activated, and this pathway is potentiated when cytokines from the TGF-β family are also present (Fig. 6, steps 2 and 3). These cytokines induce the expression of RARα, which, in the presence of retinoic acid, is activated (Fig. 6, step 4). The binding of activated RARα to the Stat3/p300/CBP/Smad complex is potentiated by the cytokines, and its binding enhances even further the transcriptional activity of the complex (Fig. 6, step 5). Although the generation of astrocytes occurs during a relatively short period of time, astrocytes are the major cell type in the brain, indicating that there is a striking, acute increase in the number of astrocytes in the perinatal period. The synergy between retinoic acid and cytokines could explain why this happens (Fig. 6, step 6). PI3K is upstream of all of these events, a fact that is consistent with previous reports from several laboratories, including ours, showing a role for various proteins from the PI3K pathway in astrogliogenesis or in the regulation of known components of the astrogliogenic pathway, such as Stat3 (14), (30,31).

GFAP is a major histological marker for reactive gliosis, an increase in astrocyte number in the adult nervous system caused by pathological conditions such as neurodegenerative disorders, stroke or injury (32). Also, some genetic disorders, such as Down’s syndrome, are associated with an increase in GFAP expression and astrocyte number (33,34). Although reactive gliosis serves as a defensive response, it can be detrimental for the recovery of damaged tissue (32), (35,36). If GFAP is required for astrocyte differentiation and viability, then the inhibition of GFAP synthesis may improve the chances for recovery from tissue damage.

Glioblastomas are the most common and deadly type of brain tumor. Their origin is still unknown, but current evidence strongly indicates that they are generated from cells with stem cell properties that proliferate and undertake incomplete, aberrant differentiation into astroglial-like cells that weakly express GFAP (6). Directing these cells to a fully, non-proliferative differentiated state could improve the effectiveness of the treatments against these types of tumors. Retinoids have, in fact, a strong antitumoral effect in glioblastoma cells both in cell cultures and patients (37-40) and this effect is mediated, at least in part, by promoting their expression of GFAP (38,39), (41).

In summary, understanding the pathways involved in GFAP expression in both precursor cells and astrocytes should allow the identification of molecular targets for manipulating these cell
types in pathological conditions. Our results indicate that approaches targeting both IL-6 and retinoic acid signaling may be a mechanism to achieve this end.
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Fig. 1. Retinoic acid receptor alpha is involved in GFAP expression. A-C, HCN-B27 adult rat hippocampal precursor cells were treated for 2 days with different combinations of gliogenic factors [All-trans-Retinoic Acid (RA, R), 1 μM; Bone Morphogenetic Protein-2 (BMP, B), 50 ng/ml; Leukemia Inhibitory Factor (LIF, L), 50 ng/ml; Fetal Bovine Serum (FBS, F), 5 %], and the expression of GFAP was determined by western blot (A) and immunocytochemistry (B-C). DAPI nuclear counterstaining was used as a reference for the total number of cells. The same concentrations and abbreviations of the gliogenic factors are used throughout the present article, unless otherwise indicated. D, Physiological concentrations of retinoic acid also induce a gliogenic response and potentiate cytokine-induced gliogenesis. E, Effects of different retinoid receptor agonists and antagonists on astrogliogenesis. RA (1 μM) and TTNPB (200 nM) are selective agonists of all RARs that do not activate RXRs; 9-cis-retinoic acid (0.5 and 1 μM) is a ligand for both RARs and RXRs; AM-580 (0.25 and 1 μM) is a specific RARα agonist; and Ro-415253 (Ro, 250 nM) is a specific RARα antagonist. Note that the films were exposed for longer times in this set of experiments in order to show the gliogenic effect of AM-580. *, significant versus control group (CON, C); §, significant versus BMP+LIF; #, significant versus retinoic acid, p<0.05.

Fig. 2. RARα is also involved in cytokine-induced GFAP expression. A, Constructs encoding luciferase under the control of serial deletions of the rat GFAP promoter and of the full length promoter with the putative RARE (AGGTCA) deleted. This putative RARE is half of a classical RARE. HCN-B27 cells were transfected with the different constructs using lipofectamine. Medium was changed 6 hours after lipofection and the cells were then treated with the cytokines and/or retinoic acid for 24 hours. All of the luciferase assays in this report are carried out following this procedure unless otherwise indicated. B, The combination of either BMP-2 or LIF with retinoic acid synergizes in the induction of transcriptional activity using the full length GFAP promoter (A1). C, The transcriptional activity induced by retinoic acid resides in the distal part of the GFAP promoter (-1546 to -1876 bp). D-E, Deletion of the putative RARE found in the GFAP promoter abolishes the transcriptional activity of retinoic acid and strongly inhibits its synergism with cytokines. F, The specific RARα inhibitor Ro-415253 (Ro, 250 nM) inhibits LIF-induced astrogliogenesis and the synergism between retinoic acid and cytokines. *, significant versus control group; a, significant versus BMP; b, significant versus LIF; #, significant versus the correspondent groups with the mutant RARE; §, significant versus the correspondent groups without Ro-415253, p<0.05.

Fig. 3. Cytokines potentiate the retinoic acid GFAP pathway. A-D, BMP-2 and LIF enhance the expression of RARα, which is necessary for retinoic acid-induced GFAP synthesis. On the other hand, retinoic acid (RA, R) has no effect on the key phosphorylation steps (Stat3 and Smad) of cytokine-induced GFAP expression. Cells were treated with retinoic acid and/or cytokines for 2 days. A, Representative western blots of whole precursor cell lysates. B-D, Densitometric analyses of the blots. E, Cytokines enhance the binding of RARα to the response element found in the rat GFAP promoter, as determined by the fluorescent DNA-protein binding assay (see Material and Methods section). F, Cytokines potentiate the transcriptional activity of retinoic receptors, as determined by a reporter construct where luciferase expression is controlled by a classic DR-5 RARE. Luciferase assays were carried out 24 hours after addition of retinoic acid and cytokines. *, significant versus control group; #, significant versus retinoic acid-treated group, p<0.05.

Fig. 4. PI3K controls the main events leading to astrogliogenesis. A, The specific PI3K inhibitor LY294002 (LY, 10 μM) completely blocks both cytokine-induced astrogliogenesis and the synergism between retinoic acid and cytokines. The specific RARα antagonist Ro-415253 (Ro, 250 nM) partially prevents cytokine-induced astrogliogenesis and the synergism between retinoic acid and cytokines. *, significant versus control group; a, significant versus BMP+LIF; b, significant versus RA, p<0.05. B-F, LY294002, but not Ro-415253, inhibits the expression of RARα and the phosphorylation of Stat3 (Ser727 and Tyr705) and Smad, steps that are essential for retinoic acid- and cytokine-induced astrogliogenesis, respectively. B, Representative blot. C-F, Densitometric analyses. All inhibitors, retinoic and cytokines were added simultaneously to cells, and proteins were extracted 2 days later. *, significant versus control group; #, significant versus the correspondent group without LY294002, p<0.05.
**Fig. 5.** PI3K controls the association between RARα, p300/CBP, Stat3 and Smad. A, knocking down the expression of p300/CBP by siRNA partially prevents the induction of GFAP expression by retinoic acid and/or cytokines and decreases the expression of RARα. Cells were transfected with lipofectamine, the medium was changed after 6 hours. Retinoic, cytokines and inhibitors were added 16 hours later, and cells were incubated with them for 2 days. B, Immunoprecipitation of p300/CBP also pulls down RARα, Stat3 and Smad proteins. The presence of BMP-2 (B) potentiates the binding of phospho-Smad to p300/CBP, but has no effect on the binding of Stat3. LIF (L) potentiates the binding of Stat3 to p300/CBP but has no effect on the binding of Smad. Both cytokines induce the binding of RARα to p300/CBP in the presence or absence of retinoic acid but this effect is much greater when retinoic acid and LIF are combined. B, Ro-415253 (Ro, 250 nM) partially inhibits the binding of RARα to p300/CBP, but has no effect on the binding of Stat3 and Smad. C, LY294002 (LY, 10 µM) strongly inhibits the binding of RARα, Stat3 and Smad proteins to p300/CBP. For the experiments described in B and C, cells were incubated with the different substances for 2 days before protein extraction and immunoprecipitation.

**Fig. 6.** Proposed revised canonical pathway leading to GFAP expression in astrocytes. 1, The promoters of the genes for GFAP and other glial markers, as well as for the key components of the canonical astrogliogenic pathway (JAK, STAT), become accessible to transcription factors and these proteins are expressed. 2 and 3, The binding of cytokines to their receptors triggers the canonical pathway. JAK, STAT and Smad proteins are phosphorylated, activated and translocated to the nucleus, where they form a transcriptional complex with p300/CBP. 4 and 5, Cytokines induce the expression of RARα, its binding to the Stat3/p300/CBP/Smad complex and to DNA. The activation of the receptor by retinoic acid may make this transcriptional complex more active and thus further potentiate the expression of the specific glial marker GFAP. 6, The synergism between retinoic acid and cytokines could explain the strong increase in the generation of astrocytes during the perinatal period of development.

**Supplementary Fig. 1.** The specific RARα antagonist Ro-415253 (Ro, 250 nM) does not inhibit the expression of RARα or the phosphorylation of Stat3 (Ser727 and Tyr705) and Smad, steps that are essential for retinoic acid- and cytokine-induced astrogliogenesis, respectively. A-D, Graphs show the densitometric analyses of the set of western blots represented by Fig. 4B.
Figure 1.
FIGURE 5

A

BMP + LIF -

RA -

 si p300

GFAP

RARα

p300

Actin

B

WB

RARα

Stat3

P-Smad

p300/CBP

C

WB

Ro

RARα

Stat3

P-Smad

p300/CBP

D

WB

LY

RARα

Stat3

P-Smad

p300/CBP

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