CCAT/enhancer-binding protein-β (C/EBPβ) is a transcription factor that plays an important role in regulating cell growth and differentiation. This protein plays a central role in lymphocyte and adipocyte differentiation and hepatic regeneration and in the control of inflammation and immunity in the liver and in cells of the myelomonocytic lineage. Our previous studies suggested that this protein could also have important functions in the brain. Therefore, we were interested in the identification of downstream targets of this transcription factor in cells of neural origin. We performed cDNA microarray analysis and found that a total of 48 genes were up-regulated in C/EBPβ-overexpressing neuronal cells. Of the genes that displayed significant changes in expression, several were involved in inflammatory processes and brain injury. Northern blot analysis confirmed the up-regulation of ornithine decarboxylase, 24p3/LCN2, GRO1/KC, spermidine/spermine N1-acetyltransferase, xanthine dehydrogenase, histidine decarboxylase, decorin, and TM4SF1/L6. Using promoter-luciferase reporter transfection assays, we showed the ornithine decarboxylase and 24p3 genes to be biological factors in cells of neural origin. We performed cDNA microarray analysis and found that a total of 48 genes have been involved in the regulation of various aspects of cell differentiation and function in multiple tissues. Six different members of the family (C/EBPα to C/EBPγ) that give rise to minimally truncated proteins have been isolated (1) and found to play a role in growth arrest and cell differentiation. Like other bZIP proteins such as c-Jun, c-Fos, and cAMP-responsive element-binding protein/activating transcription factor, C/EBPs have the ability to form homo- or heterodimers with C/EBPs and other bZIP proteins. They bind to a C/EBP-binding site, also termed the interleukin (IL)-6-responsive element, which fits with the consensus sequence T(T/G)NNGAA(T/G) (2) present in various promoters, resulting in activation or repression of transcription. C/EBPβ, also known as NF-IL6, IL-6DBP, LAP (liver-enriched activating protein), CRP2, and nuclear factor-M, is expressed in numerous tissues, including liver, adipose tissue, ovary, lung, kidney, mammary gland, and hematopoietic tissues (reviewed in Ref. 3). Transcription of the intronless C/EBPβ gene results in a single 1.4-kb mRNA that can produce at least three isoforms: full-length 38-kDa LAP1, 35-kDa LAP2, and 20-kDa LIP (liver-enriched inhibitory protein) (1, 4). Both LAP isoforms contain both the activation and bZIP domains, whereas only the later is present in LIP. LIP can therefore act as a dominant-negative inhibitor of C/EBP function by forming nonfunctional heterodimers with the other members. The C/EBPβ protein has been linked to hepatocyte-specific gene regulation because it shows high expression in liver cells (5) and binds to several control elements of liver-specific genes (4). Additionally, it was suggested that this protein contributes to the regulation of the acute-phase response of the liver (6, 7). Meanwhile, it has become clear that C/EBPβ also plays a role in other tissues. Results obtained from experiments in cell culture and with knockout mice demonstrated that C/EBPβ is very important in the process of lymphocyte (8–10) and adipocyte (11, 12) differentiation. However, much less is known about the expression and function of C/EBPβ in mammalian brain. In the invertebrate Aplysia, it has been shown that C/EBP plays an essential role in the consolidation of stable long-term synaptic plasticity (13). Several more recent studies in mammals show that C/EBPβ mRNA is widely expressed in adult mouse brain (14) and that this protein could be implicated in long-term synaptic plasticity and memory consolidation in rat hippocampus (15). Also, our previous study (16) showed that the overexpression of C/EBPβ in a mouse neuroblastoma cell line induces neuronal differentiation, and Mennard et al. (17) have shown that activation of C/EBPβ promotes the generation of neurons versus astrocytes in progenitor cells isolated from embryonic mouse cortex. Altogether, these results suggest that the transcription factor C/EBPβ could have important functions in the brain. Obviously, knowledge of the network of genes altered by C/EBP in the brain is required to fully understand the physiological functions of C/EBP in this organ. To identify C/EBPβ downstream targets in this study, we compared the gene ex-
pression of two stable C/EBPβ-transfected clones (C22 and CE) obtained from a neuroblastoma cell line (18) with vector alone-transfected cells (CB) using cDNA microarrays. In 10 replicate experiments, the expression levels of 48 genes were up-regulated in both clones C22 and CE. Our experiments also revealed that C/EBPβ induction is highly correlated with the expression of many genes involved in inflammatory processes in the brain and that the expression of this protein is induced after a neuronal injury. Thus, our data suggest the possibility that C/EBPβ plays a role in the regulation of the processes that follow brain injury through hitherto unknown mechanisms.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfections

Mouse TR cells (18) were propagated and maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) containing 10% fetal bovine serum, 40 μg/ml gentamicin, and 2 mM glutamine at 37 °C and 5% CO2. For the selection of stably transfected cells, 0.5 × 106 cells were seeded into a 6-cm diameter tissue culture plate, incubated overnight, and transfected with 3 μg of pZeoSV2-C/EBPβ using the calcium phosphate precipitation technique as described previously (16). Twelve hours after transfection, the medium was replaced with regular growth medium, and the cells were incubated for 24 h, after which they were subcultured at 1:10 dilution with the addition of Zeocin (350 μg/ml). The growth medium was renewed every 3 days, and fresh Zeocin was added. Individual colonies were transferred into 24-well plates, expanded, and screened for C/EBPβ expression.

For transient transfection experiments (24β) and ornithine decarboxylase (ODC) promoter constructs, semiconfluent cells were transfected by the calcium phosphate precipitation technique in 12-well plates. After 8 h of exposure to the Cβ2-DNA mixture, cells were washed with buffer A (20 mM Tris-HCl (pH 7.4), 1 mM Na2HPO4, 140 mM NaCl, and 5 mM KCl), incubated for another 24 h in complete medium, and harvested for determination of luciferase and chloramphenicol acetyltransferase.

Firefly luciferase

pLuc-Inv

Renilla luciferase

pLucR

We obtained a 15,247-mouse cDNA preparation of the mouse C/EBPβ using the calcium phosphate precipitation technique as described previously (16). Twelve hours after transfection, the medium was replaced with regular growth medium, and the cells were incubated for 24 h, after which they were subcultured at 1:10 dilution with the addition of Zeocin (350 μg/ml). The growth medium was renewed every 3 days, and fresh Zeocin was added. Individual colonies were transferred into 24-well plates, expanded, and screened for C/EBPβ expression.

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Firefly luciferase

pLuc-Inv

Renilla luciferase

pLucR

Total RNA was extracted from clones CB, C22, and CE by homogenization in guanidine thiocyanate as described previously (19). Probe for microarray hybridization were generated using 25 μg of total RNA, which was first annealed with 4 μg of oligo(dT)12-18 primer (Invitrogen) at 70 °C for 10 min. Afterward, cDNA synthesis was carried out at 42 °C for 2 h in buffer containing 60 units of Superscript II RNAse H− reverse transcriptase (Invitrogen); 100 μM dNTP mixture in water as described previously (19). Each transient transfection experiment was repeated at least three times in triplicate.

The mouse 24β promoter was PCR-amplified from mouse genomic DNA using high fidelity Platinum® Taq DNA polymerase (Invitrogen). The primers used were 5′-TAT ATG GAT CCA ATG AAA GCA GCC ACA TCT GAG-3′ (forward sequence) and 5′-TTC TTA TGA GCA GAG GAA GAG CAG AGG-3′ (reverse sequence). They were designed according to the published sequence of the mouse 24β promoter (GenBank®/EBI accession number X81627). PCRs were performed according to the manufacturer’s recommendations, and the amplification product was cloned and sequenced in both directions. The entire promoter fragment (~719 to +18, p24β) was subcloned in the promoterless luciferase reporter vector pXPl. The construct pOCCLux1 (containing 1.5 kb of the rat ODC promoter) was a gift of Dr. A. P. Butler (Anderson Cancer Center, University of Texas (20)).

Preparation of the ‘Mouse 15K cDNA Array’

Generation of PCR Products—We obtained a 15,247-mouse cDNA clone set from NIA, National Institutes of Health. The bacteria were subcultured, and then the clone inserts (sizes of 0.5–3 kb) were amplified by PCR using the Expand high fidelity system (Roche Applied Science) together with universal plasmid primers. The 5′-primer was C12 amino modified. Additionally, different control PCR fragments were prepared from organisms foreign to mouse (Table I). Purification of the PCR fragments was performed in a 96-well format on a Biomet FX robot (Beckman Coulter, Fullerton, CA) based on a silica gel matrix system (Nucleospin Robot 96-B extract kit, Macherey Nagel, Dueren, Germany). Subsequently, aliquots of the eluates were run on 192-well agarose gels (1%) to check the size, quality, and concentration of the PCR fragments. Around 500 PCRs had to be improved in a second run. For spotting, aliquots of the purified PCR fragments were transferred from 96- to 384-well plates, and 12 × SSC buffer (1.8 M NaCl and 0.18 M sodium citrate (pH 7.0)) was added to a final 2-fold concentration.

Spotting and Immobilization—PCR fragments were spotted onto amino-silanized glass slides. Spotting was performed on an OmnisGrid Spotter (GeneMachines, San Carlos, CA) using 32 Telechem split needles (SMP3). Controls were spotted in various dilutions (undiluted, 1:2, 1:4, and 1:8) into each subgrid; control 2 was placed into every subgrid, whereas controls 1 and 3–5 were distributed into distinct subgrids. Spotting was performed at 21 °C with 40% relative humidity. For attachment of the DNA to the surface, slides were subsequently incubated at 50 °C in a humid chamber for 3.5 h and then at 100 °C for 10 min. Until usage, they were stored in a dry dark place.

Isolation of RNA, Labeling of cDNA Probes, and Hybridization to cDNA Arrays

Total RNA was extracted from clones CB, C22, and CE by homogenization in guanidine thiocyanate as described previously (19). Probes for microarray hybridization were generated using 25 μg of total RNA, which was first annealed with 4 μg of oligo(dT)12-18 primer (Invitrogen) at 70 °C for 10 min. Afterward, cDNA synthesis was carried out at 42 °C for 2 h in buffer containing 60 units of Superscript II RNAse H− reverse transcriptase (Invitrogen); 100 μM dNTP mixture in water as described previously (19). Each transient transfection experiment was repeated at least three times in triplicate.

Hybridizations were carried out in a 40-μl volume hybridization mixture that included final concentrations of 50% formamide, 6× SSC, 0.5% SDS, 5× Denhardt’s solution, 0.1 μg/ml salmon sperm DNA, and 0.25 μg/ml poly(dA). The mixture was applied to the array, which was covered with a 24 × 60-mm glass coverslip and placed in a sealed chamber to prevent evaporation. The microarrays were then incubated at 42 °C for 16 h. Hybridized slides were washed by shaking once for 10 min in 0.1× SSC and 0.1% SDS and twice for 5 min in 0.1× SSC at room temperature. Washed slides were centrifuged and analyzed as described below.

Data Analysis

Microarray slides were scanned using a Gene Pix 4000B simultaneous dual wavelength scanner (Axon Instruments, Inc., Union City, CA), and the data obtained were analyzed using Chip Skipper analysis software. For data integration, a segmentation-based spot/background detection was performed for each spot separately. Data normalization was carried out in two steps analyzing logarithmic intensity (base 2) (Supplemental Fig. S2).

Northern Blot Analysis

Northern blot analysis was performed as described previously (19). Briefly, 20 μg of total RNA were electrophoresed on a 2.2% formaldehyde/1% agarose gel in 1× MOPS buffer at 100 V for 3–4 h and transferred to nylon membranes (Biodyne, Pall Corp.). Labeled probes were used to detect the same probe by using random primers and hybridized with the membranes for 20 h at 42 °C (formamide, 3× SSC, and 0.2% SDS). Methylene blue staining of the membranes were used as a loading control. The cDNAs used as probes for 24p3L/CNC2, GRO1/12C, spermine/spermine N1-acetyltransferase, xanthine dehydrogenase, histidine decarboxylase, decorin, and TM4SF1/L6 antigen were obtained by re-
verse transcription-PCR analysis using the Superscript one-step reverse transcription-PCR kit (Invitrogen) and the primers described in Table II. The cDNA for ODC was a gift of Dr. P. Coffino (Department of Microbiology and Immunology, University of California, San Francisco, CA) (21).

**Immunoblot Analysis**

TR cells were lysed in phosphate-buffered saline containing 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and protease inhibitors and spun at 12,000 × g for 20 min, and the supernatants were removed and stored at −70 °C until analysis. Equal amounts of protein (20 μg) were electrophoresed on 10% SDS-polyacrylamide gels and blotted onto nitrocellulose membranes. After transfer, immunoblotting with a polyclonal antibody to rat C/EBPβ was carried out as described previously (16). The polyclonal anti-cyclooxygenase (COX)-2 and monoclonal anti- α-tubulin antibodies were obtained from Santa Cruz Biotechnologies (Santa Cruz, CA) and Sigma, respectively. Values in text are the average of the quantification of at least three independent experiments corresponding to three different samples.

**Analysis of the Intracellular Localization of C/EBPβ by Confocal Microscopy**

CB, C22, and CE cells were plated on glass coverslips in 24-well cell culture plates and grown in regular medium for 24 h. The cells were then washed, fixed for 10 min with methanol at −20 °C, and permeabilized with 0.1% Triton X-100 for 30 min at 37 °C. After a 1-h incubation with the primary antibody (16), cells were washed with phosphate-buffered saline and incubated with a fluorescein-labeled secondary antibody for 45 min at 37 °C. Subcellular localization was determined using a TCS SP2 laser-scanning spectral confocal microscope (Leica Microsystems). The images were obtained using a series of 0.5-μm (depth) spaced cell fluorescent slices (z axis).

**Scratch-Wound Model**

Confluent monolayers of clones CB, C22, and CE were wounded by scratching with a plastic pipette (yellow) tip along lines at right angles to each other. Cultures were then rinsed with buffer A, maintained in regular medium for the indicated times, and processed for Western blot and immunocytochemical analyses as described above.

**RESULTS**

To investigate the cellular action of the C/EBPβ protein in neuronal cells, we stably introduced a pZeoSV2 vector encoding C/EBPβ into TR cells (see “Experimental Procedures”), and different Zeocin-resistant clones were tested for C/EBPβ expression using an antibody specific for C/EBPβ (16). In subsequent experiments, two independent clones (C22 and CE) were used, with empty pZeoSV2 vector-transfected TR cells (CB) used as control cells. The immunoblot in Fig. 1A shows that the content of the C/EBPβ protein was very low in parental TR cells, and its levels were markedly increased in clones C22 and CE. We next performed immunofluorescence analysis to study the intracellular localization of the C/EBPβ protein in the different clones. The confocal images shown in Fig. 1B show that, in control cells, there was almost no signal, but clones C22 and CE presented a strong immunofluorescence, which was localized mainly in the nucleus and distributed in multiple bright foci.

To analyze global gene expression in C/EBPβ-overexpressing neuronal cells, cDNA microarray hybridizations were performed using an NIA 15,247-mouse cDNA clone set (22). The cDNA probes derived from clones CB and C22 or clone CE were fluorescently labeled with Cy3-dUTP (green) or Cy5-dUTP (red), respectively. These probes were applied simultaneously onto the microarray, and the two fluorescent images were scanned with a fluorescence laser-scanning device (Supplemental Fig. S1). Red and green fluorescent signals indicated genes whose expression levels were relatively higher in cells overexpressing C/EBPβ and the backbone vector, respectively. The differential expression of each gene was calculated from the relative intensity of the Cy5 versus Cy3 fluorescent signal. Ten independent experiments were conducted comparing vector-transfected and C/EBPβ-overexpressing cells. Fig. 2 shows one representative plot of the differential expression of the 15,247 genes in one of the ten experiments. Overall, the expression of most genes was not altered by C/EBPβ. Other plots are shown in Supplemental Fig. S2.

A total of 48 genes in clones C22 and CE had a differential expression value compared with vector-transfected cells in all the experiments and are considered up-regulated by C/EBPβ (Table III and Supplemental Table). The group of genes up-regulated by C/EBPβ was further analyzed on the basis of functional similarity using the DRAGON Database. As shown in Table III, these genes can be clustered into several groups, as genes involved in the immune/inflammatory response, metabolism, signal transduction, extracellular and membrane proteins, intracellular transport and cytoskeleton, protein degradation, and others. Some of these cDNA sequences also correspond to expressed sequence tags for which the full-length sequence is not available in public domain data bases and to unknown genes. It is of note that many of the up-regulated genes encode proteins involved in inflammatory and brain injury processes.

In view of the data commented on above and to verify the results of the microarray experiments, we performed Northern blot analysis on RNA isolated from CB, C22, and CE cells using several cDNA probes encoding genes involved in inflammation and brain injury that were shown to be up-regulated by C/EBPβ in Table III. As shown in Fig. 3, the mRNA levels of ODC, 24p3/LCN2, GRO1/KC, xanthine dehydrogenase, histidine decarboxylase, decorin, and TM4SF1/L6 antigen showed a significant induction in C22 and CE cells compared with control CB cells. The induced expression of spermidine/spermine N1-acetyltransferase was observed only in C22 cells.

The strongest C/EBPβ-induced increase in expression was seen for the 24p3 gene, which belongs to the lipocalin family of proteins (23). Lipocalins are small secreted proteins that play a role in diverse biological processes through binding of small hydrophobic molecules, interaction with cell-surface receptors, and formation of macromolecular complexes (24). The 24p3 protein has been implicated in processes such as programmed cell death and inflammatory response (25, 26).

*3 Available at pevsnerlab.kennedykrieger.org/dragon.htm.
also shows a significant increase in expression (27). ODC is one of the key enzymes in the polyamine biosynthetic pathway catalyzing the formation of putrescine from ornithine. Interestingly, there are numerous data showing an involvement of ODC in brain injury (28–30), and neuropathological and clinical studies have demonstrated that the ODC/polyamine system is heavily involved in various human brain diseases (31, 32). Other genes also involved in inflammatory processes and brain injury were induced by C/EBPβ, including the chemokine Gro1 (33–35); regulatory enzymes such as histidine decarboxylase (36–39) and xanthine dehydrogenase (40–44); an enzyme implicated in polyamine metabolism, spermidine/spermine N1-acetyltransferase (45); as well as two genes (decorin and Tm4sf1/L6 antigen) involved in structural integrity (46–49) (Table III).

To test whether the C/EBPβ protein regulates transcription of the 24p3 and ODC genes, we next performed transient transfection experiments with the reporter plasmids pODClux1m (20) and p24p3lac, containing 1.2 and 0.8 kb, respectively, of the promoter regions of the ODC and 24p3 genes. As shown in Fig. 4A and 5A, the reporter activities of both constructs were significantly enhanced: 3.4-fold (24p3 gene) and 1.6-fold (ODC gene) by cotransfection with an expression vector for C/EBPβ. We next tested 24p3 and ODC promoter activities in CB (control), C22, and CE cells (Figs. 4B and 5B). In these experiments, we also noted a very strong increase in luciferase activity in cells stably expressing the C/EBPβ protein: 25-fold (C22) and 13-fold (CE) increases with the 24p3 promoter construct and a 2-fold (C22 and CE) increase with the pODClux1m construct. These data are in agreement with the higher levels of 24p3 and ODC mRNAs observed in these clones.

We next studied the possible involvement of C/EBPβ in the process of neuronal injury. To this end, we used an in vitro model of neuronal reaction to injury. The model we utilized here was first introduced by Yu et al. (50) and has been widely used since to analyze neural reactions to lesions (51, 52). Experimental lesions were made by scratching of clones CB, C22, and CE, and the expression of C/EBPβ was explored at different times after scratching by Western blot and immunofluorescence analyses. As shown in Fig. 6A, the levels of C/EBPβ protein in clones CB, C22, and CE started to increase between 1 and 3 h after scratching and remained elevated 6 h after in clones C22 and CE. The increase in the levels of C/EBPβ protein after injury was more pronounced in clones overexpressing C/EBPβ. At 3 h, the levels of C/EBPβ in clones CB, C22, and CE increased by 1.3-, 2.0-, and 2.4-fold, respectively, compared with basal (0 h) values. These results were further confirmed by immunocytochemical analysis. The confocal images in Fig. 6B show that, in clone C22, the C/EBPβ signal was significantly induced 1 and 3 h after lesion.

Finally, we analyzed whether the increased expression of C/EBPβ was associated with induction of the expression of COX-2, an enzyme that plays a prominent role in many forms of inflammation. Western blot analysis using a COX-2-specific antibody showed that, under basal conditions, the COX-2 protein could be detected in control CB cells (Fig. 7A), and its content was significantly higher in clones C22 and CE. Three hours after injury, a transient increase could be observed in CB cells (1.3-fold) (Fig. 7B), which was more evident in clones C22 (1.8-fold) and CE (1.5-fold) (3 and 6 h after injury, respectively) and which was coincident with the peak in C/EBPβ expression observed in these clones after lesion.

DISCUSSION

Although the effects of C/EBPβ have been investigated in detail in cell types such as adipocytes, hepatocytes, and lymphocytes (53–57), very few target genes of this transcription factor have been identified in the central nervous system. In this study, we report the expression profiling of the transcription program controlled by C/EBPβ in neuroblastoma cells using cDNA microarrays. Our study identified several genes whose expression was significantly up-regulated by the overexpression of C/EBPβ in neuronal cells. A particularly interesting group among the genes up-regulated by C/EBPβ is one that encodes proteins involved in inflammation and brain injury. Northern analysis confirmed the changes in ornithine decarboxylase, 24p3/LCN2, GRO1/KC, spermidine/spermine N1-acetyltransferase, xanthine dehydrogenase, histidine de-
carboxylase, decorin, and TM4SF1/L6 antigen. In addition, we have shown that the levels of C/EBPβ were increased after a neuronal lesion together with the levels of COX-2 enzyme. These data suggest that C/EBPβ may contribute to regulation of brain injury.

It is known that C/EBPβ plays an important role in regulating several aspects of inflammation and immunity in the liver and in cells of the myelomonocytic lineage (58). Indeed, C/EBPβ was originally identified because of its inducibility by IL-6 in human hepatoma cells. It has been subsequently determined by many independent studies that C/EBPβ and C/EBPδ are strongly up-regulated at the transcriptional level by inflammatory stimuli such as turpentine oil and bacterial lipopolysaccharide and by recombinant cytokines such as IL-6, IL-1, and tumor necrosis factor-α (reviewed in Ref. 59). Inflammation is an important part of the pathophysiology of traumatic brain injury and chronic neurodegenerative disorders. Evidence now suggests that syndromes such as multiple sclerosis and Alzheimer’s and Parkinson’s diseases and traumatic brain injury have important inflammatory and immune components and may be amenable to treatment by anti-inflammatory and immunotherapeutic approaches (reviewed in Ref. 60). The key players in these processes are the numerous immune mediators released within minutes of the primary injury. Inflammatory cytokines such as tumor necrosis factor-α, IL-1, and IL-6 appear to be robustly activated and secreted as early as 1 h after ischemic and traumatic insults (61–63).

The results presented here show a very strong induction of 24p3, a member of the lipocalin family of small secreted polypeptides that is present in several tissues, including the brain (26), and it is thought to be involved in inflammatory processes. Lipocalins have remarkably diverse functions, including retinal transport, olfaction, pheromone transport, and prostaglandin synthesis. Recently, several reports have implicated members of this family in inflammatory processes, and it has been shown that the lipocalin proteins bind various ligands important in homeostasis and inflammation. For example, plasma levels of 24p3 are elevated during the acute-phase response (26), which involves a massive expansion of neutrophils. Also, it has been shown that the human homolog of 24p3 (neutrophil-associated lipocalin, NGAL) (24), which is present in neutrophilic granules, can play a role in the neutrophilic
apoptosis that follows an inflammatory response.

Another gene shown in this study to be strongly up-regulated by C/EBPβ is ODC. The ODC and spermidine/spermine \( N^2 \)-acetyltransferase genes are also involved in inflammatory processes and several disorders of the nervous system. ODC is one of the key enzymes in the polyamine biosynthetic pathway catalyzing the formation of putrescine (the precursor of the natural polyamines spermidine and spermine) from ornithine (27). On the other hand, both polyamines are converted back to natural polyamines spermidine and spermine by the action of spermidine/spermine \( N^2 \)-acetyltransferase. The ODC protein is localized in many different processes and several disorders of the nervous system. ODC is one of the key enzymes in the polyamine biosynthetic pathway catalyzing the formation of putrescine (the precursor of the natural polyamines spermidine and spermine) from ornithine (27). 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On the other hand, both polyamines are converted back to natural polyamines spermidine and spermine by the action of spermidine/spermine \( N^2 \)-acetyltransferase. The ODC protein is localized in many different brain areas, including the hypothalamus, some cortical areas, hippocampus, cerebellar cortex, some cranial nuclei, and nucleus ruber (29), and central nervous system injury results in an increase in the activities of both ODC and spermidine/spermine \( N^2 \)-acetyltransferase (64). Natural polyamines are of considerable importance for the developing and mature nervous systems, and it has also become clear that the polyamine system is involved in various brain pathological events such as traumatic brain injury, stroke, Alzheimer’s disease, and others (reviewed in Ref. 65). In this study, we have also shown that C/EBPβ could regulate the expression of the ODC and 24p3 genes. We chose the ODC and 24p3 genes because their expression was strongly induced in C/EBPβ transfectants and because sequence analysis of their promoter regions revealed the presence of several potential C/EBPβ-binding sites. The demonstration that ODC and 24p3 promoter activities were increased by C/EBPβ suggests that both genes could be direct downstream targets of this transcription factor in neuroblastoma cells. In addition to the ODC, 24p3, and spermidine/spermine \( N^2 \)-acetyltransferase genes, other genes involved in inflammatory processes in the brain identified in this screen include histidine decarboxylase, xanthine dehydrogenase, Gro1, decorin, and
after ischemia (66). GRO1 is a protein that belongs to the CXC subfamily of chemokines, which are involved in the recruitment of leukocytes to sites of inflammation. Also, some reports suggest that GRO1 may play important roles in the brain. GRO1 induces tau phosphorylation in mouse primary cortical neurons, and an increase in its expression has been detected in the brains of post-mortem human brain tissues, suggesting a possible involvement of this gene in neurodegenerative disorders (35, 67). Decorin is a small proteoglycan present in the extracellular matrix, and it is thought to behave as a growth inhibitor protein in different cell types and to regulate the NF-κB pathway. A role of decorin in inflammation of the nervous system and in Alzheimer’s disease has been also reported (46, 68), and its levels are significantly up-regulated after brain injury (47). Finally, TM4SF1/L6 antigen is a protein that is structurally related to the tetraspanin family of proteins, which are found associated with different integrins (69). Although the cellular role of TM4SF1 is not well established, there are several reports implicating some members of the tetraspanin protein family in neurite extension and outgrowth (70). Northern blot analysis of all these genes confirmed that they are up-regulated by C/EBPβ in neuroblastoma cells. This observation and the fact that the promoters of histidine decarboxylase, xanthine dehydrogenase, GRO1, and decorin contain putative binding sites for C/EBPβ strongly suggest that they are downstream targets of C/EBPβ; and therefore, this protein could represent an important new pathway in the regulation of their expression in neuronal cells.

All together, given the important role of ODC, 24p3, GRO1, xanthine dehydrogenase, and decorin in brain inflammatory processes, the observed gene expression changes in TR neuroblastoma cells differentially expressing C/EBPβ are consistent with the hypothesis that C/EBPβ plays an important role in the
transcriptional control of brain injury processes. Consistent with the notion that C/EBPβ could be involved in inflammatory processes in the brain, it has been reported recently that lipopolysaccharide, IL-1β, and tumor necrosis factor-α induce the mRNA levels of C/EBPβ in mouse primary astrocytes (71).

In this work, we have also shown an injury-induced activation of C/EBPβ expression in clones overexpressing this protein in an in vitro scratch-wound model. Scratching the monolayer of C22 cells resulted in a significant increase in C/EBPβ protein levels throughout all the monolayer. Neuronal cells in confluent monolayers are coupled to each other, and junctional connections render a quick intercellular communication possible throughout large culture areas. Therefore, injury to a confluent monolayer of cells was expected to affect not only the directly wounded cells, but also larger populations in the dish. Signals spreading through the intercellular junctions or via released factors (72) could have resulted in the observed effect after scratching of C22 cells.

Finally, we also observed up-regulation of COX-2 in clones C22 and CE (compared with vector-transfected cells) as well as a transient induction coincident with the increase in C/EBPβ protein levels after wound injury. COX-2 is the rate-limiting enzyme for the conversion of arachidonic acid to prostaglandins and a key therapeutic target for the treatment of brain injury. Inflamatory processes associated with the increased expression of COX-2 and elevated levels of prostaglandin E₂ have been implicated in the cascade of events leading to neurodegeneration in a variety of pathological settings (73–75). COX converts arachidonic acid to prostaglandin H₂, the precursor of prostaglandin E₂ and several others prostanoids, and exists in

![FIG. 6. Expression of the C/EBPβ protein in CB, C22, and CE cells after wound-induced injury.](image)

**A.** Western blot analysis. Cells were lysed 1, 3, 6, 12, and 24 h after scratching, and cellular proteins were immunoblotted with anti-C/EBPβ or anti-α-tubulin antibody. **B.** confocal imaging of the localization of C/EBPβ in clone C22. Cells were grown on glass coverslips, fixed with cold methanol, and processed for immunofluorescence using the same anti-C/EBPβ antibody as in A.

![FIG. 7. Expression of the COX-2 protein in CB, C22, and CE cells growing under normal conditions (A) or after wound-induced injury (B).](image)

Cells were lysed 1, 3, 6, 12, and 24 h after scratching, and cellular proteins were immunoblotted with anti-COX-2 or anti-α-tubulin antibody.
eukaryotic cells in two main isoforms: COX-1, which is constitutively expressed in many cell types, and COX-2, which is normally not present in most cells, but whose expression can readily be induced in inflamed tissues (76). In this regard, it is noteworthy that the expression of the COX-2 promoter is induced by COXβ and that this protein plays an obligatory role in COX-2 expression in macrophages (77). Although both isoforms synthesize prostaglandin H2, COX-1 is primarily involved in the production of prostanooids relevant to physiologic processes, whereas COX-2 is mainly responsible for the production of prostanooids linked to pathological events (76). Therefore, the data presented here showing an increase in COX-2 protein levels in C/EBPβ-overexpressing cells and after neuronal injury again implicate C/EBPβ in inflammatory processes in neuronal cells.

Collectively, our data provide evidence for C/EBPβ up-regulation in brain injury and support a role for C/EBPβ in transcriptional regulation of inflammatory processes in the brain. This results of this study suggest that regulation of C/EBPβ may be a valuable target for the development of new therapies for brain disorders involving inflammatory processes. Clearly, further studies are required to verify this hypothesis.

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