BASIC FIBROBLAST GROWTH FACTOR STIMULATES PHOSPHORYLATION OF CCAAT/ENHANCER-BINDING PROTEIN δ BY ACTIVATION OF MITOGEN-ACTIVATED PROTEIN KINASE

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ABSTRACT

Stimulation of cultured astrocytes with basic fibroblast growth factor (bFGF) increased phosphorylation of CCAAT/enhancer-binding protein (C/EBP) δ , but not C/EBP β . The maximal phosphorylation was obtained 60 min after stimulation. PD098059, a specific inhibitor of mitogen-activated protein kinase kinase (MAP kinase kinase), inhibited phosphorylation of C/EBP δ . Immunohistochemistry showed that bFGF stimulation of cultured astrocytes induced the translocation of activated MAP kinase from the cytoplasm to the nucleus and surrounding regions. This translocation was inhibited by addition of PD098059. Dibutyl cyclic AMP (dbcAMP) increased the total amount of C/EBP δ protein but had no effect on its phosphorylation state. KN93, a specific inhibitor of Ca²⁺/calmodulin-dependent protein kinase II, and calphostin C, a specific inhibitor of protein kinase C, did not inhibit phosphorylation of C/EBP δ following stimulation with bFGF. Gel mobility shift analysis using nuclear extracts from bFGF-treated cells showed increases in C/EBP site binding activities 60 min after stimulation with bFGF. These results suggest that bFGF stimulates phosphorylation of C/EBP δ through activation of MAP kinase, and is involved in stimulation of gene expression by phosphorylation of C/EBP δ .

The CCAAT/enhancer-binding protein (C/EBP) family members are transcription factors belonging to a class of basic region-leucine zipper proteins (29, 50, 56). The C/EBP family consists of at least six members, including C/EBP α (30), β (1, 14, 39, 53), γ (43), δ (24, 25, 53), ϵ (53) and CHOP-10 (44). Functions of members of the C/EBP family have been extensively studied in liver cell nuclei; some C/EBP proteins are involved in

expression of acute phase genes induced by mediators of inflammation (2, 9, 10, 14, 39, 41, 42). In PC12 cells, expression of the *c-fos* gene is regulated by C/EBP β (33), and the C/EBP family is involved in differentiation and proliferation of the cells (56). The transcriptional activity of C/EBP β was reported to be increased by phosphorylation (11, 31, 34, 35, 47-49, 52).

We have reported that stimulation of cultured astrocytes with glutamate activates Ca²⁺/calmodulin-dependent protein kinase II (CaM kinase II) with concomitant phosphorylation of cytoskeletal proteins (12, 17, 37, 55). Furthermore, both C/EBP β and C/EBP δ but not C/EBP α are expressed in astrocytes (8, 17, 54). After exposure to glutamate, C/EBP β mRNA levels increased within 10 min, while C/EBP δ mRNA levels decreased by 6 h. In astrocytes, glutamate stimulation resulted in phosphorylation of C/EBP β , most likely by CaM kinase II (54). Stimulation of astrocytes

Abbreviations: bFGF, basic fibroblast growth factor; C/EBP, CCAAT/enhancer-binding protein; CaM kinase II, Ca²⁺/calmodulin-dependent protein kinase II; cAMP, cyclic AMP; dbcAMP, dibutyl cyclic AMP; KRH, Krebs-Ringer HEPES; MAP kinase, mitogen-activated protein kinase; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TBS, Tris-buffered saline; MEK, mitogen-activated protein kinase kinase

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with glutamate increased the DNA-binding activities of C/EBP β and δ (54).

Basic fibroblast growth factor (bFGF) is a member of a family of heparin-binding growth factors which activate a receptor tyrosine kinase (21, 46). In the brain, bFGF has important roles in development and regeneration of tissue (5, 20, 38, 51). It has a broad action on both glial (20, 38) and neuronal cells (51) and is involved in brain repair following traumatic injury (15). It was recently reported that C/EBP family members are immediate early genes required for the consolidation of long-term facilitation in *Aplysia* (3).

Growth factors including bFGF activate mitogen-activated protein kinase (MAP kinase) via activation of receptor tyrosine kinases in various cells (6, 13, 22, 23, 26, 28, 45). We reported that bFGF activates MAP kinase in association with an increase in DNA synthesis in astrocytes (27).

The present study shows that stimulation of astrocytes with bFGF phosphorylates C/EBP δ , but not C/EBP β by activation of MAP kinase.

MATERIALS AND METHODS

Materials

The following chemicals and reagents were obtained from the indicated sources: fetal calf serum, JRH Biosciences; [32 P]orthophosphate, ICN Biochemicals; [γ - 32 P]ATP, DuPont NEN; L-glutamate, basic fibroblast growth factor (bFGF) and dibutyryl cAMP (dbcAMP), Sigma; Hanks' balanced salts, Life Technologies; calphostin C, Kyowa Medex; protein A-Sepharose CL-4B, Pharmacia Fine Chemicals; Eagle's minimal essential medium, Nissui Pharmaceutical; anti-C/EBP β antibody and anti-C/EBP δ antibody, Santa Cruz Biotechnology; PD098059, Research Biochemicals International; KN93, Seikagaku Kogyo; anti-active MAP kinase antibody, Promega; fluorescein-conjugated goat affinity-purified antibody to rabbit IgG, ICN Pharmaceuticals.

Cell Culture

Cultured astrocytes were prepared from the neocortices of 1-day-old Wistar rats, as described previously (55). The brain cortices from 1-day-old rats were mechanically dissociated by trituration with fire-polished Pasteur pipettes and plated in 80-cm² flasks in growth medium. The growth medium consisted of 45% Eagle's minimum essential medium, 45% Hanks' balanced salt solution and 10% fetal

calf serum. The cultures were maintained at 37°C in a water-saturated atmosphere of 95% air and 5% CO₂, with the medium replaced twice a week. After 1 week, contaminating cells were removed by orbital shaking (32). The astrocytes were subcultured in 35-mm, 60-mm or 100-mm dishes coated with poly-L-lysine and maintained for at least 5 days before use. Astrocytes were almost flat and polygonal and were classified as type 1 astrocytes (40). Neurons were never observed in subcultured cells.

Labeling of Cells and Phosphorylation of C/EBP β , and C/EBP δ in Astrocytes

Cultured astrocytes were grown to confluency and incubated in serum-free medium for 24 h. The astrocytes in 60-mm dishes were washed once with phosphate-free and serum-free minimum essential medium and labeled in 2.0 ml of the medium containing carrier-free [32 P]orthophosphate (0.25 mCi/ml), as described previously (18). After labeling for 5 h, the cells were preincubated in Krebs-Ringer HEPES buffer (KRH) containing 128 mM NaCl, 5 mM KCl, 1 mM sodium phosphate, 1.2 mM MgSO₄, 10 mM glucose, 2.7 mM CaCl₂, and 20 mM HEPES (pH 7.4), at 37°C for 15 min. After preincubation, the cells were incubated without (control) or with bFGF (100 ng/ml) for the indicated times. In assays requiring inhibitors, cells were preincubated in KRH or an inhibitor in KRH at 37°C for 15 min. After preincubation, the cells were incubated without (control) or with bFGF (100 ng/ml), an inhibitor or bFGF plus each inhibitor for 60 min. Stimulation of cells was terminated by aspiration of the medium, and the cells were immediately frozen in liquid N₂. Frozen astrocytes were scraped from a 60-mm dish and homogenized with 0.9 ml of a buffer containing 50 mM HEPES, 0.1% Triton X-100, 4 mM EGTA, 10 mM EDTA, 15 mM Na₄P₂O₇, 100 mM β -glycerophosphate, 25 mM NaF, 0.4 M NaCl, 50 μ g/ml leupeptin, 50 μ g/ml trypsin inhibitor, and 25 μ g/ml pepstatin A. Triton X-100-soluble fractions were then subjected to immunoprecipitation with the anti-C/EBP β antibody (5 μ g) plus 50 μ l of protein A-Sepharose CL-4B (50%, v/v) or the anti-C/EBP δ antibody (5 μ g) plus 50 μ l of protein A-Sepharose CL-4B (50%, v/v) at 4°C overnight, as previously described (19). The immunocomplex immobilized on protein A-Sepharose CL-4B was washed three times with homogenization buffer. Each immunoprecipitate

was eluted from protein A Sepharose CL-4B by boiling in SDS sample buffer for 4 min. Supernatants were further subjected to SDS-PAGE in 12% acrylamide, followed by autoradiography. A Bio-Imaging analyzer (model BA100) was used to quantify the amounts of ^{32}P -incorporation into C/EBP β or C/EBP δ .

Indirect Immunofluorescence Analysis

Astrocytes cultured in 35-mm dishes were incubated in serum-free medium for 24 h. The cells were preincubated in KRH at 37°C for 15 min, or preincubated with 50 μM PD098059 in KRH for 50 min. After preincubation, the cells were incubated without (control) or with bFGF (100 ng/ml) or bFGF plus PD098059 for 60 min. Incubation of cells was terminated by aspiration of the medium. The cells were washed with phosphate-buffered saline (PBS) and fixed at -20°C for 10 min with cold methanol. After a 10-min dehydration at room temperature, the cells were treated with 0.01% Triton X-100 in PBS for permeabilization. Nonspecific antibody binding was blocked by preincubation with 5% goat serum in PBS (blocking solution), followed by incubation overnight with the anti-active MAP kinase antibody in the blocking solution at a 50-fold dilution. The cells were then washed five times with PBS and incubated for 60 min with a fluorescein-conjugated goat affinity-purified antibody to rabbit IgG in PBS containing 0.01% Triton X-100. Finally, the cells were washed with PBS, and mounted in Tris-buffered saline (TBS) containing 50% (v/v) glycerol, 0.15 M NaCl and 20 mM Tris-HCl (pH 7.4). The cells were observed and photographed with a FLU-OVIEW BX50 (Olympus).

Immunoblotting

Cultured astrocytes were grown to confluency and incubated in serum-free medium for 24 h. Astrocytes in 100-mm dishes were preincubated in KRH or dbcAMP (1 mM) in KRH for 15 min. After preincubation, the cells were incubated without (control) or with bFGF (100 ng/ml), dbcAMP (1 mM) or bFGF plus dbcAMP for 60 min. Stimulated astrocytes were washed twice with the cold buffer containing 0.15 M NaCl, 1.5 mM KH_2PO_4 , 3 mM KCl, 10 mM $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 0.5 mM EDTA, scraped with 1 ml of the same buffer at 0°C, collected from the dish, and centrifuged at $2,500 \times g$ for 5 min. The pellets were homog-

enized with 0.1 ml of a buffer containing 50 mM HEPES, 0.1% Triton X-100, 4 mM EGTA, 10 mM EDTA, 15 mM $\text{Na}_4\text{P}_2\text{O}_7$, 100 mM β -glycerophosphate, 25 mM NaF, 0.4 M NaCl, 50 $\mu\text{g}/\text{ml}$ leupeptin, 50 $\mu\text{g}/\text{ml}$ trypsin inhibitor, and 25 $\mu\text{g}/\text{ml}$ pepstatin A. Triton X-100-soluble fractions were incubated for 15 min on ice and centrifuged at $15,000 \times g$ for 10 min. Aliquots of supernatants were assayed for protein concentration, and the same amount of protein (25 μg) was subjected to SDS-PAGE in 12% acrylamide and transferred electrophoretically to a nitrocellulose membrane at 60V for 3 h. After the membrane was incubated at 4°C for 1 h with 4.5% skim milk in TBS (pH 7.4) to block nonspecific binding sites, it was incubated with antibodies overnight, as previously described (55). The anti-C/EBP β and anti-C/EBP δ antibodies were diluted 1:100 in TBS containing 2.5% BSA. Immunoreactive peptides were detected using ^{125}I -labeled protein A.

Preparation of Nuclear Extracts and Gel Mobility Shift Assay

Cultured astrocytes were grown to confluency and incubated in serum-free medium for 24 h. Astrocytes in 100-mm dishes were preincubated in KRH or PD098059 (50 μM) in KRH for 15 min. After preincubation, the cells were incubated without (control) or with bFGF (100 ng/ml) or bFGF plus PD098059 for 60 min. The cells were washed twice, homogenized in cold TBS, and centrifuged at $1,500 \times g$ for 15 min. The pellets were resuspended in TBS and centrifuged at $15,000 \times g$ for 15 s. The pellets were resuspended in 400 μl of the buffer containing 10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, and allowed to swell on ice for 15 min. Then 25 μl of 10% NP-40 was added, and the suspension was vigorously vortexed for 10 s and centrifuged for 30 s. The pellets were resuspended in 50 μl of the buffer containing 20 mM HEPES (pH 7.9), 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT and 1 mM PMSF, allowed to swell on ice 10 min and centrifuged for 5 min. The supernatants were used for a gel mobility shift assay as nuclear extracts. The probe used in gel-shift assays was the double-stranded oligonucleotide corresponding to the CaM kinase II-responsive element (CaMRE) of the Rous sarcoma virus long terminal repeat promoter (Rous sarcoma virus nucleotides -231 to -193; 5'-AAATGTAGTCTTATGCAATA-CACTTGTAGTCTTGCAACA-3'). This sequence

constitutes a binding site for C/EBP (52). The 5' ends of probe DNAs were labeled with [γ - 32 P] ATP by T4 polynucleotide kinase. The standard binding reaction was carried out in 10 μ l of a mixture containing 25 mM HEPES (pH 7.6), 50 mM KCl, 1 mM EDTA, 0.5 mM spermidine, 0.6 mM dithiothreitol, 12% glycerol, 5 μ g of poly(dI-dC), 1 fmol of the 32 P-labeled probe (about 2×10^4 dpm), and nuclear extracts (3–4 μ g of protein). In competition analysis, 1 pmol of the competitor oligonucleotide was mixed before the addition of nuclear extracts. After 30 min on ice, 2.5 μ l of 20% Ficoll were added, and the samples were loaded onto a 5% polyacrylamide gel in a buffer containing 89 mM Tris, 89 mM boric acid, and 2.5 mM EDTA. Electrophoresis was performed at 10 V/cm for 2 h at room temperature. The gel was then dried and autoradiographed on X-ray films at -80°C . For antibody double shift analysis, 3 μ g of the anti-C/EBP β or the anti-C/EBP δ antibody was added to mixtures of the binding reaction for the last 15 min.

Other Methods

Protein concentration was determined by the method of Bradford (7) with bovine serum albumin as the standard.

Statistical Evaluation

Values are means $\pm$ SE. The significance of the difference between values was calculated by Student's *t* test, and *p* values of < 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

Phosphorylation of C/EBP β and C/EBP δ by Stimulation with bFGF in Cultured Astrocytes

Phosphorylation of C/EBP β and C/EBP δ following bFGF stimulation of astrocytes was examined in a time course (Fig. 1). During the time period examined, phosphorylation of C/EBP δ increased, while phosphorylation of C/EBP β remained unchanged. Consistent with previous findings, C/EBP δ migrated at approximately 33 kDa (8, 11, 54). Also observed were bands of 42 and 44 kDa, which may represent unrelated proteins cross-reacting with C/EBP antibody (11). Phosphorylation of C/EBP δ maximally increased by approximately 64% 60 min after stimulation and decreased by 120 min. Increase in phosphorylation was not observed as

Fig. 1 Effects of bFGF on phosphorylation of C/EBP β and C/EBP δ in cultured astrocytes during a time course. Cultured astrocytes were incubated in serum-free medium for 24 h. After cells were labeled with [32 P]orthophosphate (0.25 mCi/ml) for 5 h, then were incubated in KRH for 15 min and stimulated without (control) or with bFGF (100 ng/ml). Immediately after the medium was aspirated at indicated times, the cells were frozen in liquid N₂. The cells were then scraped and homogenized with buffer. Triton X-100-soluble fractions were subjected to immunoprecipitation with anti-C/EBP β or anti-C/EBP δ antibody. A: After 60 min of stimulation, phosphorylated C/EBP β and C/EBP δ were immunoprecipitated with the anti-C/EBP β antibody (5 μ g) or the anti-C/EBP δ antibody (5 μ g) and were analyzed by SDS-PAGE, followed by autoradiography. B: The time course of phosphorylation of C/EBP β or C/EBP δ following stimulation with bFGF. The radioactivity was quantified by a Bio-Imaging analyzer BA100 (Fuji Photo Film, Japan) and expressed as a percentage of control. Values are means $\pm$ SE. The increase in phosphorylation of C/EBP δ at 60 min and 120 min were statistically significant versus control (*n* = 5). *, *P* < 0.05, versus the control

late as 30 min after stimulation. This observation suggests that it is unlikely that protein kinase C, cAMP-kinase or CaM kinase II is involved in phosphorylation, since phosphorylation of C/EBP β by CaM kinase II increases within 5 min of stimulation (16, 17, 54, 55).

Fig. 2 Effect of MEK inhibitor PD098059 on bFGF-stimulated phosphorylation of C/EBP δ in cultured astrocytes. Cultured astrocytes were incubated in serum-free medium for 24 h and labeled with [³²P]orthophosphate (0.25 mCi/ml) for 5 h as in Fig. 1. The cells were preincubated without or with PD098059 (50 μ M) in KRH for 15 min, and incubated without (control) or with bFGF (100 ng/ml), PD098059 (50 μ M) or bFGF plus PD098059 for 60 min. After incubation, the medium was aspirated and the cells were frozen in liquid N₂. The cells were then scraped and homogenized with buffer. Triton X-100-soluble fractions were subjected to immunoprecipitation with the anti-C/EBP δ antibody. A: Phosphorylated C/EBP δ was immunoprecipitated with the anti-C/EBP δ antibody (5 μ g) and analyzed by SDS-PAGE, followed by autoradiography. B: ³²P-incorporation into C/EBP δ was determined by a Bio-Imaging analyzer (model BA100) and expressed as a percentage of control. Values are means $\pm$ SE (n = 3). *, $P < 0.05$, versus the control

Effects of glutamate on phosphorylation of C/EBP β and C/EBP δ were examined in astrocytes during a time course. In contrast to the results with bFGF, glutamate stimulated phosphorylation of C/EBP β , as previously reported (17, 54), but not of C/EBP δ in the 120-min time period. Maximal phosphorylation of C/EBP β following stimulation with 500 μ M glutamate was observed in 5 min, with an approximately 45% increase over the control (54).

Effects of a MAP Kinase Kinase Inhibitor on Phosphorylation of C/EBP δ by Stimulation with bFGF

Phosphorylation of C/EBP δ by stimulation with bFGF suggests that MAP kinase may be involved in its phosphorylation, because the bFGF receptor contains an intracellular tyrosine kinase domain and stimulation of the receptor induces activation of the MAP kinase cascade (13, 22, 23, 26–28). Furthermore, the time course of phosphorylation is sustained for a relatively longer period compared to second messenger-dependent protein kinases such as protein kinase C, cAMP-kinase and CaM kinase II.

PD098059, a specific inhibitor of mitogen-activated protein kinase or extracellular signal-regulated kinase kinase (MEK) (4, 36), inhibited stimulation of C/EBP δ phosphorylation by bFGF (Fig. 2). PD098059 by itself had no effect on phosphorylation of C/EBP δ . These results suggest that MAP kinase phosphorylates C/EBP δ .

Effects of a MAP Kinase Kinase Inhibitor on Activation and Translocation of MAP Kinase to the Nucleus with bFGF

We examined the localization of MAP kinase in cultured astrocytes following treatment with bFGF and PD098059. MAP kinase was exclusively localized in the cytosol of untreated astrocytes (Fig. 3A). Strong immunostaining was observed in the nucleus and the area surrounding the nucleus in astrocytes stimulated with 100 ng/ml bFGF (Fig. 3B). The bFGF-induced increase in nuclear immunoreactivity of MAP kinase was totally abolished by treatment with bFGF plus PD098059 (Fig. 3C). These findings demonstrate that PD098059 interferes with bFGF stimulated activation and translocation of MAP kinase from the cytosol to the nucleus. The blockade of the translocation is correlated with inhibition of both MAP kinase activation and C/EBP δ phosphorylation.

Effects of dbcAMP on Phosphorylation of C/EBP δ by Stimulation with bFGF

We previously reported that dbcAMP inhibited basal MAP kinase activity in cultured astrocytes as well as MAP kinase activity stimulated by bFGF (27). Therefore, we examined whether dbcAMP inhibits phosphorylation of C/EBP δ .

dbcAMP alone did not stimulate phosphoryla-

Fig. 3

Fig. 4 Effects of dbcAMP on phosphorylation of C/EBP δ induced by bFGF in cultured astrocytes. Cultured astrocytes were incubated in serum-free medium for 24 h and labeled with [32 P]orthophosphate for 5 h as in Fig. 1. The cells were preincubated without or with dbcAMP (1 mM) in KRH for 15 min, and incubated without (control) or with dbcAMP (1 mM), bFGF (100 ng/ml) or bFGF plus dbcAMP for 60 min. After incubation, the medium was aspirated and the cells were frozen in liquid N $_2$. The cells were then scraped and homogenized with buffer. Triton X-100-soluble fractions were subjected to immunoprecipitation with the anti-C/EBP δ antibody. A: Phosphorylated C/EBP δ was immunoprecipitated with the anti-C/EBP δ antibody (5 μ g) and analyzed by SDS-PAGE, followed by autoradiography. B: 32 P-incorporation into C/EBP δ was determined by a Bio-Imaging analyzer (model BA100) and expressed as a percentage of control. Values are means $\pm$ SE (n = 7). *, $P < 0.05$, versus the control

Fig. 3 Translocation of MAP kinase by treatment with bFGF and its inhibition with PD098059. Cultured astrocytes were incubated in serum-free medium for 24 h. The cells were preincubated in KRH for 15 min, or with PD098059 (50 μ M) in KRH for 50 min, and incubated without (control) (A) or with bFGF (100 ng/ml) (B) or bFGF plus PD098059 (C) for 60 min. The cells were fixed in cold methanol, followed by treatment with 0.01% Triton-X. Fixed cells were incubated with the anti-active MAP kinase antibody and processed for indirect immunofluorescence. $\times 200$

tion of C/EBP δ in cultured astrocytes (Fig. 4), nor did it have a significant effect on bFGF stimulated phosphorylation of C/EBP δ (Fig. 4).

To understand discrepancies between the present and previous (27) reports, the effect of dbcAMP on the level of C/EBP δ protein was examined (Fig. 5). dbcAMP increased the level of C/EBP δ protein, while bFGF did not (Fig. 5B), suggesting that dbcAMP stimulates protein synthesis of C/EBP δ . Addition of bFGF plus dbcAMP increased significantly the levels of C/EBP δ protein over control, but to a lesser degree than did dbcAMP alone.

Taken together with above results, dbcAMP was assumed to decrease phosphorylation of C/EBP δ by inhibition of MAP kinase. However, the level of C/EBP δ protein increased following treatment with dbcAMP. Thus, inhibition of phosphorylation of C/EBP δ was not apparently observed.

Effects of Inhibitors on Phosphorylation of C/EBP δ

To identify kinases involved in phosphorylation of C/EBP δ , the effects of inhibitors such as KN93 and calphostin C were examined. KN93, a specific inhibitor of CaM kinase II, had no effect on phosphorylation of C/EBP δ by stimulation with bFGF (Fig. 6). Similar results were obtained with calphostin C, a specific inhibitor of protein kinase C. As shown in Fig. 7, calphostin C alone and together with bFGF had no inhibitory effect on phosphorylation of C/EBP δ .

These results indicate that CaM kinase II and protein kinase C are not involved in phosphorylation of C/EBP δ .

Effects of bFGF Treatment on DNA-Binding Activity

We examined effects of bFGF on DNA-binding activities of C/EBP family members in cultured astrocytes by gel mobility shift assay. After incubation without or with bFGF or bFGF plus PD098059 for 60 min, nuclear extracts from the astrocytes were prepared. Following exposure to bFGF, the binding activities of C/EBPs apparently increased, and two complexes, C-1 and C-2, were observed (Fig. 8A). Following the exposure to bFGF plus PD098059, the binding activities of C/EBPs decreased in both complexes (Fig. 8, A and B). The specificity of the DNA-protein complexes C-1 and C-2 was confirmed by competition with a nonlabeled homologous oligonucleotide

Fig. 5 Effects of bFGF and dbcAMP on protein levels of C/EBP δ in cultured astrocytes. Cultured astrocytes were incubated in serum-free medium for 24 h. The cells were preincubated without or with dbcAMP (1 mM) in KRH for 15 min, and incubated without (control) or with bFGF (100 ng/ml), dbcAMP (1 mM) or bFGF plus dbcAMP for 60 min. A: Immunoblot analysis was carried out with the anti-C/EBP δ antibody. B: The relative amount of C/EBP δ on the autoradiograph was determined by a Bio-Imaging analyzer (model BA100) and expressed as a percentage of control. Values are means $\pm$ SE (n = 5). *, $P < 0.05$, versus the control

(Fig. 8A). Treatment of the DNA-protein complexes with anti-C/EBP β or C/EBP δ antibody led to an apparent supershift of the complexes with a concomitant decrease in the C-1 and C-2 bands (Fig. 8A). The addition of the anti-C/EBP β antibody decreased the intensity of the C-1 complex stronger than the C-2 complex (Fig. 8A, lane 6), while the C-1 and C-2 complexes both diminished by addition of the C/EBP δ antibody (Fig. 8A, lane 7). These results suggest that C-1 and C-2 complexes induced in response to bFGF are heterooligomers containing at least two proteins each related to C/EBP β and C/EBP δ and that the phosphorylation of C/EBP δ with bFGF increases DNA-binding activity.

The present study showed that stimulation of astrocytes with bFGF increases phosphorylation of C/EBP δ , but not C/EBP β , by MAP kinase. Furthermore, phosphorylation of C/EBP δ increased its

Fig. 6 Effects of KN93, an inhibitor of CaM kinase II, on phosphorylation of C/EBP δ induced by bFGF. Cultured astrocytes were incubated in serum-free medium for 24 h and labeled with [³²P]orthophosphate for 5 h as in Fig. 1. The cells were preincubated without or with KN93 (20 μ M) in KRH for 15 min, and incubated without (control) or with bFGF (100 ng/ml), KN93 (20 μ M) or bFGF plus KN93 for 60 min. After incubation, the medium was aspirated and the cells were frozen in liquid N₂. The cells were then scraped and homogenized with buffer. Triton X-100-soluble fractions were subjected to immunoprecipitation with the anti-C/EBP δ antibody. A: Phosphorylated C/EBP δ was immunoprecipitated with the anti-C/EBP δ antibody (5 μ g) and analyzed by SDS-PAGE, followed by autoradiography. B: ³²P-Incorporation into C/EBP δ was determined by a Bio-Imaging analyzer (model BA100) and expressed as a percentage of control. Values are means $\pm$ SE (n = 4). *, $P < 0.05$, versus the control

DNA-binding activities. A report by Wegner *et al.* (52) shows increased transcriptional activity of C/EBP β following glutamate stimulation; here we show that bFGF increases DNA binding of C/EBP δ , which may in turn result in increased transcriptional activity. Such activities may be involved in functions of astrocytes, such as proliferation.

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Fig. 7 Effects of calphostin C, an inhibitor of protein kinase C, on phosphorylation of C/EBP δ induced by bFGF. Cultured astrocytes were incubated in serum-free medium for 24 h and labeled with [32 P]orthophosphate for 5 h as in Fig. 1. The cells were preincubated without or with calphostin C (100 nM) in KRH for 15 min, and incubated without (control) or with bFGF (100 ng/ml), calphostin C (100 nM) or bFGF plus calphostin C for 60 min. After incubation, the medium was aspirated and the cells were frozen in liquid N₂. The cells were then scraped and homogenized with buffer. Triton X-100-soluble fractions were subjected to immunoprecipitation with the anti-C/EBP δ antibody. A: Phosphorylated C/EBP δ was immunoprecipitated with the anti-C/EBP δ antibody (5 μ g) and analyzed by SDS-PAGE, followed by autoradiography. B: 32 P-Incorporation into C/EBP δ was determined by a Bio-Imaging analyzer (model BA100) and expressed as a percentage of control. Values are means $\pm$ SE (n = 4). *, $P < 0.05$, versus the control

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Fig. 8 Changes in DNA-binding activities of C/EBP family members following treatment with bFGF. Cultured astrocytes were incubated in serum-free medium for 24 h. A: The cells were preincubated in KRH or with PD098059 (50 μ M) in KRH for 15 min, and incubated without (control) (C) or with bFGF (100 ng/ml) (F) or bFGF plus PD098059 (P) for 60 min. The cells were collected and nuclear extracts were prepared. A gel-shift assay using a 32 P-labeled oligonucleotide probe was performed as described in Materials and Methods. Protein-DNA complexes were separated on a 5% non-denaturing polyacrylamide gel, followed by autoradiography. (-) denotes 32 P-labeled probe in the absence of nuclear extract, and (H) indicates the addition of a 1,000-fold excess of unlabeled probe used as a competitor. (β) and (δ) indicate binding assays done in the presence of C/EBP β antibody (3 μ g) and C/EBP δ antibody (3 μ g), respectively. C-1 and C-2 mark specific complexes which could be eliminated by competing oligonucleotide probe; the asterisk marks a nonspecific complex. B: 32 P-Incorporation into the complexes C-1 and C-2 was determined by a Bio-Imaging analyzer (model BA 100) and expressed as a percentage of control. Values are means $\pm$ SE (n = 4). *, $P < 0.05$, versus the control

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