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Basic Fibroblast Growth Factor Modulates the Expression of PDZ Domain-containing Proteins in Cultured Cortical Neurons

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Summary. **Different cytokines and growth factors, together with their receptors, are expressed in brain tissue. One such molecule is the basic fibroblast growth factor (bFGF) that has recently been shown to promote survival following insults to neurons *in vivo* or *in vitro*. In this study, we found that repeated treatment of neocortical cultures with bFGF modulated the expression of various PDZ domain-containing proteins (SAP97, GRIP1, Pick1, and PSD-93) and that the patterns of their immunostaining matched the bFGF effects on their total protein expression. For instance, bFGF decreased the expression of SAP97, GRIP1, and Pick1 (PDZ proteins that interact with the AMPA-type glutamate receptor subunits GluR1 and GluR2/3). PSD-93, which associates with the NMDA-type glutamate receptor, was increased by bFGF. Moreover, the interactions of GluR1 with SAP97 and GluR2 with GRIP1 were down-regulated by the repeated bFGF stimulation, as revealed by co-immunoprecipitation. Together, these results describe a novel function of bFGF in the regulation of expression of PDZ proteins.**

Key words—basic fibroblast growth factor, cytokines, glutamate receptor, PDZ domain, cortex.

INTRODUCTION

Basic fibroblast growth factor (bFGF) was initially characterized as a peptide factor affecting the growth and survival of various cell types. Recently, however, more versatile functions have been ascribed to bFGF on neurons. These include the promotion of neuronal and stem cell survival, increasing neurite sprouting and growth, acute modulation of synaptic transmission, and a potential role as a neuroprotective agent against acute stroke and degenerative diseases of the brain[1-9]. bFGF has also been implicated in cell-fate determination and in migration of developing neurons[10-15].

PDZ domain-containing proteins such as PSD-93, SAP102, SAP97, GRIP1, and Pick1 are involved in the subcellular dynamics of ionic channels and receptor proteins to/from the cell surface[16-19], or in the enhancement/stabilization of receptor protein expression[20,21]. The AMPA-type glutamate receptors (GluR1-4) mediate the fast excitatory neurotransmission in the vast majority of brain synapses and their addition to, or removal from, the synapse have been correlated with important physiological phenomena such as long-term potentiation and depression (LTP and LTD), respectively[18,22-25]. Some PDZ proteins

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Abbreviations—AMPA, alpha-amino-3-hydroxy-5-methyl-4-isoxazoleproprionic acid; bFGF, basic fibroblast growth factor; DMEM, Dulbecco's-modified Eagle medium; EDTA, ethylenediaminetetraacetic acid; FBS, Fetal bovine serum; GluR, AMPA-type glutamate receptor subunit; GRIP1, Glutamate receptor-interacting protein 1; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; LTD, Long term depression; LTP, Long term potentiation; NMDA, N-methyl-D-aspartate, PAGE; poly-acrylamide gel electrphoresis; PDZ protein, PSD-95, Dlg1; ZO-1 domain-containing protein; Pick1, Protein interacting with C kinase; PSD-93, Postsynaptic density protein 93 kDa; SAP97, Synapse-Associated Protein 97 kDa; SAP102, Synapse-Associated Protein 102 kDa; SDS, Sodium dodecyl sulfate.

cluster K^+ channels as well[26-28]. Little is known, however, about the regulation of PDZ protein expression by cytokines, growth factors, and neurotrophins. Factors that affect the expression of PDZ proteins will potentially lead to a change in the subcellular distribution of their interacting proteins such as AMPA receptors and K^+ channels[21,26]. We chronically applied bFGF to neocortical neuronal cultures and investigated its effects on the expression of various PDZ proteins and the outcome of their regulation on the AMPA-type GluR1 and GluR2 receptor subunits.

MATERIALS AND METHODS

Neuronal cultures

Pregnant Sprague-Dawley rats were purchased from SLC Ltd. (Shizuoka, Japan), and cerebral neocortices of day 18 embryos were dissociated with papain (1 mg/ml) and plated onto poly-D-lysine-coated dishes or chamber slides (Permanox, Lab-Tek, Nunc, Naperville, IL) at a low to medium cell density (800–1200 cells/mm^2) into Dulbecco's-modified Eagle medium (DMEM) containing 10% calf serum. Dissociated cells were allowed to attach to the coated surfaces for 1 h. They were then transferred into fresh DMEM containing 0.5 mM pure glutamine (Ajinomoto, Tokyo, Japan), 2% fetal bovine serum (FBS), nutrient mixture N2, and 10 mM HEPES (pH7.3), where they were maintained overnight. The following day, the medium was replaced with serum-free N2 DMEM. This procedure reduced glial contamination to less than 5% of the total cells[29]. Untreated control cultures or cultures that were supplemented daily with purified human recombinant bFGF (20 ng/ml; Sigma Chemicals, St. Louis, MO) for 4 d were used for all subsequent experiments.

Immunoblotting

Cultured neurons were harvested with sample buffer [10% glycerol, 2% sodium dodecyl sulfate (SDS), 65 mM Tris-HCl; pH 7.5]. Total cell lysates or immunoprecipitated proteins (see below) were denatured by boiling in 3x sample buffer containing 0.1 M dithiothreitol for 5 min, separated by SDS-polyacrylamide gel electrophoresis (PAGE) with 4/20 polyacrylamide slab gels (Daiichi Pure Chemicals, Tokyo), and blotted to nitrocellulose membranes. The membranes were incubated with primary antibodies (see below) at 4℃ overnight. Immunoreactivity was detected using goat anti-rabbit (DAKO, Kyoto, Japan) or goat anti-mouse (Jackson ImmunoResearch Laboratories, West Grove, PA) antibodies conjugated to peroxidase (diluted 1 : 10000) followed by chemiluminescence reaction (ECL kit; Amersham, Uppsala, Sweden) combined with film exposure. Primary antibodies used in this study were as follows: Anti-SAP97 monoclonal (1 μg/ml; StressGen Biotechnologies, San Diego, CA), anti-GRIP1 (1 μg/ml; Upstate Biotechnology, New York, NY), anti-SAP102, anti-PSD-93[30], (both 1 μg/ml; gifts from Dr. M. Watanabe), anti-Pick1[19] (2 μg/ml), anti-C-terminal GluR1[29] (1 μg/ml), anti-GluR2/3 (1 μg/ml; Chemicon, Temecula, CA), and anti-β-Actin (1 μg/ml; Boehringer-Mannheim, Indianapolis, IN) antibodies. The specificity of the immunoreactivity was determined by comparing the size of the detected bands to those of the reported molecular weights[16,20,31-34].

Immunostaining

Cultured neurons were washed with phosphate-buffered saline and fixed for 30 min with 4% paraformaldehyde in a 0.1 M phosphate buffer (pH 7.3). Neurons were immunostained with the anti-GluR1 C-terminus antibody (15 μg/ml), anti-GluR1 N-terminus antibody[35], (5 μg/ml), anti-SAP97 (10 μg/ml; StressGen Biotech.), anti-PSD-93 (5 μg/ml), anti-GRIP1 (1 μg/ml Upstate Biotech.), anti-Pick1 (5 μg/ml) or anti-PanPDZ (0.5 μg/ml; Transduction Laboratories, San Jose, CA) antibodies. Immunoreactivity was revealed using the diaminobenzidine method and visualized with the aid of a Zeiss microscope (Axioskop) fitted with LCD color camera (DP50-CU; Olympus Co., Tokyo). All pictures were taken with a 20x objective, 1.25x zoom at 1/250 s shutter speed using Studio Lite software (Pixera Corp., Los Gatos, CA).

Immunoprecipitation

Cultured neurons were lysed with immunoprecipitation buffer (10 mM Tris-HCl, 140 mM NaCl, 1 mM EDTA, and 1% deoxycholate; pH 7.5) supplemented with protease and phosphatase inhibitors (10 U/ml leupeptin, Wako Pure Chemicals, Osaka, Japan; 0.5 mM NaF, 0.05 mM polymethylsulfonylfluoride, 1 μg/ml pepstatin A, and 1 mM Na_3VO_4, Sigma Chemicals). Immunoprecipitation was performed as previously indicated[36]. Typically, 3–5 μg of anti-N-terminal GluR1[35], or anti-N-terminal GluR2 (Chemicon) antibodies, which had been pre-adsorbed to Protein A/Protein G- Sepharose beads, were incubated with 200 μg of total cell lysate at 4℃ overnight, washed with a binding buffer and subjected to immunoblotting as indicated above.

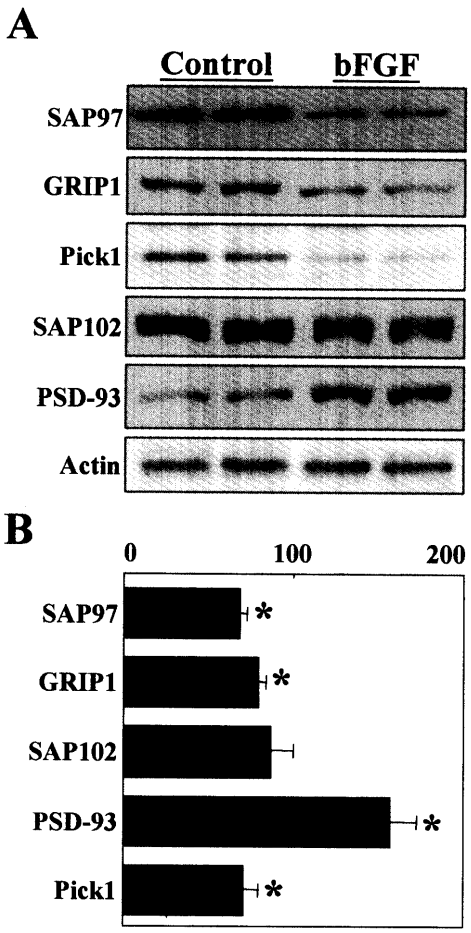

Fig. 1. bFGF alters the expression of PDZ proteins in neocortical cultures. Control or bFGF-treated cultures were lysed and equal protein amounts were probed with antibodies specific for the AMPA receptor-associated PDZ domain-containing proteins SAP97, GRIP1 and Pick1, for another two PDZ proteins, SAP102 and PSD-93, as well as for β-actin as a control. Representative results show that bFGF treatment resulted in a generalized decrease in various PDZ proteins, with the exception of PSD-93 that was instead increased (**A**). The magnitudes of the bFGF-mediated change in the levels of expression of these proteins were quantified by densitometry and the results are shown in (**B**). *: $p<0.05$ with the Mann-Whitney U-test (n=8 cultures).

RESULTS

Regulation of PDZ protein expression by bFGF

Total cellular protein was obtained from neocortical neurons grown in culture for 5 days in the presence or absence of bFGF (added daily for 4 days; 20 ng/ml) and used for immunoblotting to investigate the effects of this cytokine on the expression of various PDZ proteins such as SAP97, GRIP1, Pick1, SAP102 and PSD-93 (Fig. 1A). The total protein yields from 10 cm culture dishes were similar in the control and bFGF-stimulated cultures: 1.50 ± 0.34 mg/dish for control cultures, and 1.59 ± 0.42 mg/dish for bFGF-treated cultures (n=7, p=0.19). Densitometric quantification of the bands from four duplicate cultures revealed that their intensities were significantly different in the bFGF-treated samples from the untreated controls (total n=8, Fig. 1B). For instance, bFGF decreased the amounts of native SAP97, GRIP1, and Pick1. The SAP102 protein level in the bFGF-treated sample was not significantly different from that of the control. However, the total protein expression of PSD-93 was increased following the long-term incubation with bFGF.

Effects of bFGF on PDZ protein immunostaining

Neocortical neuronal cultures, which had been grown with or without bFGF, were fixed with 4% paraformaldehyde, and primary antibodies for the PDZ proteins were added overnight at the concentrations indicated in Materials and Methods. The density of the cells was determined in control and bFGF-stimulated cultures: 1092 ± 305 cell/mm^2 in control cultures and 1280 ± 333 cell/mm^2 in bFGF-treated cultures (p=0.162). The results showed that the intensity of the immunoreactivity as well as the number of strongly labeled cells were indeed different between the bFGF-treated and control cultures (Fig. 2): The percentage of densely stained cells decreased or increased matching the effects of bFGF on the total protein levels. For instance, GRIP1-like immunoreactivity decreased in the bFGF treated cultures as compared with the control (Fig. 2A and D). Pick1-like and SAP97-like immunostaining decreased in the bFGF-treated cultures as well (data not shown). Conversely, yet in accordance with the immunoblotting results, PSD-93-like immunoreactivity was enhanced by bFGF (Fig. 2B and E). Furthermore, incubating fixed cultures with an antibody that recognizes a scope of PDZ domains common to various PDZ proteins[37] revealed a decrease in the amount of labeling in the bFGF-treated cultures as well as in the number of intensely stained cells as compared with the untreated controls (Fig. 2C and F). Moreover, this last result further suggests that the negative effect of bFGF on the expression of PDZ proteins is rather generalized despite the PSD-93 exception. Densely stained cells were counted and their percentage over the total number of cells determined and plotted (Fig. 2G).

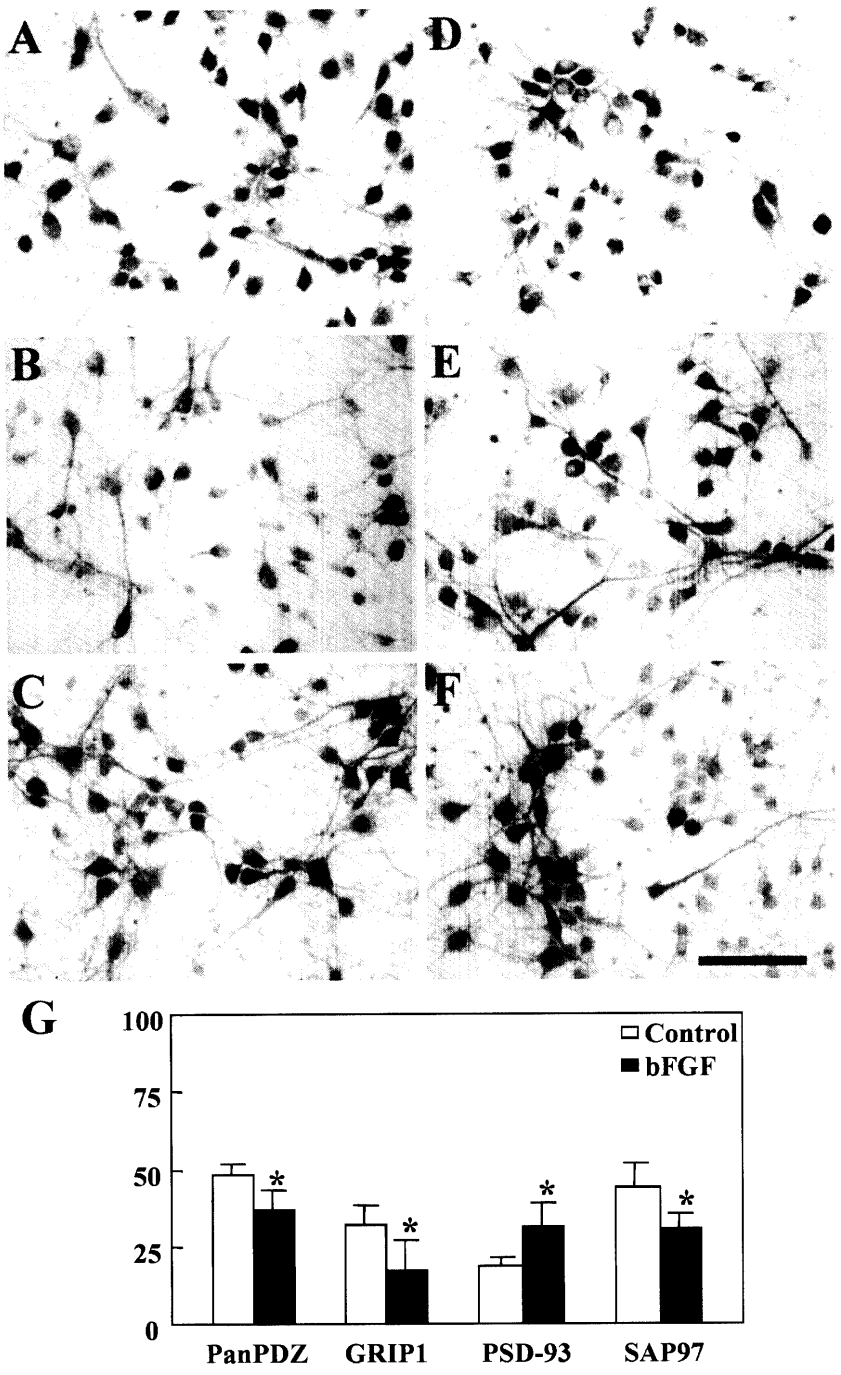

Fig. 2. Immunohistochemistry of PDZ proteins in control (**A**, **B** and **C**) or bFGF-treated (**D**, **E** and **F**) neuronal cultures. Dissociated cortical neurons were either stimulated with bFGF or not for 4 days, and fixed and incubated with antibodies specific to the GRIP1 (**A** and **D**), PSD-93 (**B** and **E**) proteins or with a PanPDZ (**C** and **F**) antibody. The PanPDZ antibody recognizes PDZ domains shared by many postsynaptic density proteins and the results show a decrease in the number of intensely stained cells in the bFGF-treated cultures. In parallel with the immunoblotting results, bFGF-treated cultures had a lower number of cells densely stained for GRIP1 but a higher number of those stained for PSD-93. (**G**) The percentage of intensely positive cells was determined from 5 different fields of each condition in two sister cultures. *: $p<0.05$ by Student's t-test. Scale bar, 50 μm.

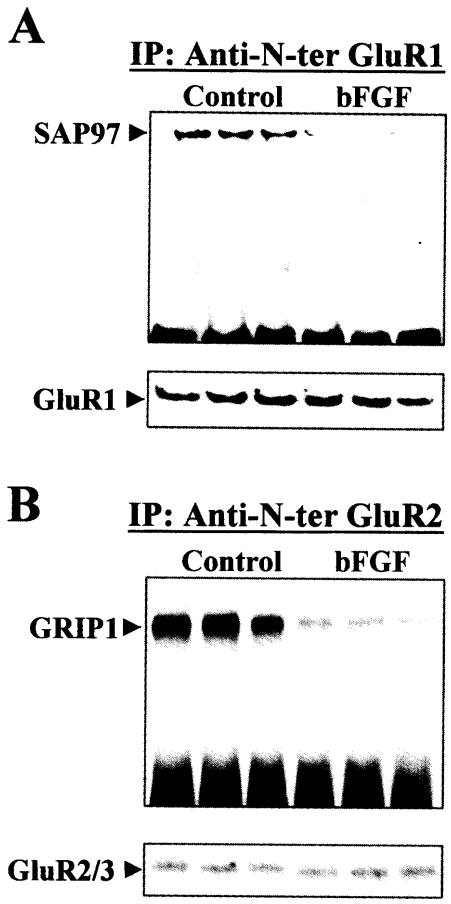

Fig. 3. bFGF decreases the binding of AMPA-type glutamate receptors to PDZ proteins. Day 5 cortical cultures, which were either treated with bFGF for 4 days or not, were lysed in an immunoprecipitation buffer. Equal amounts of proteins were incubated overnight with ProteinA/ProteinG Sepharose beads on which antibodies that recognize the N-termini of GluR1 or GluR2 had been preadsorbed. Representative results of immunoblotting with anti-SAP97 and anti-GRIP1 revealed that the total amounts of SAP97 and GRIP1 respectively, associated with GluR1 and GluR2 were decreased by prior treatment with bFGF (both $p<0.001$ by Student's t-test). Lower panels in (**A**) and (**B**) are controls showing GluR1 and GluR2 proteins as probed with the anti-C-termini of GluR1 and GluR2, respectively. The experiment was repeated twice with triplicate sister cultures of each condition (total n=6).

bFGF treatment reduced the interaction of AMPA-receptor subunits with their associated PDZ proteins

In order to assess, at least partially, the outcome of PDZ protein regulation by bFGF, co-immunoprecipitation experiments were undertaken such that anti-N-terminal GluR1 or anti-N-terminal GluR2 antibodies were used to pull down interacting PDZ proteins from control or bFGF-stimulated cultures. The immunoprecipitation results revealed that the levels of SAP97 and GRIP1 respectively interacting with GluR1 and GluR2 were decreased by bFGF treatment as compared with the control (Fig. 3A and B; upper panels). The same membranes were re-probed with anti-C-terminal GluR1 and anti-C-terminal GluR2 antibodies and the results are shown in the lower panels (Fig. 3A and B), illustrating that equal amounts of GluR1 and GluR2 reacted with the specific anti-N-terminal antibodies and implying that bFGF did not affect the total amounts of GluR1 and GluR2 proteins proteins.

bFGF enhanced GluR1-like immunostaining with the C-terminal antibody

A previous report from our laboratory showed that daily treatment of neocortical neuronal cultures with bFGF did not change the expression of the GluR1 subunit[29]. To confirm the effects of bFGF on the interaction between GluR1 and SAP97, we attempted to immunostain cultured cortical neurons with an anti-C-terminal GluR1 antibody (Fig. 4, upper and middle panels). Assuming that the C-terminus of GluR1 is liberated from its binding to SAP97, the epitope recognized by the antibody becomes unmasked and consequently the immunoreactivity should be elevated. The results revealed that the percentage of intensely stained cells with the anti-C-terminus GluR1 antibody was indeed increased in the bFGF-treated cultures (8.6%±4.3 in the control culture, 21.7%±8.0 in bFGF-treated cultures n=7; p=0.007). Dense GluR1-like staining with the C-terminal antibody in bFGF-treated neurons was cytoplasmic and appeared in the neuritic processes (Fig. 4, middle panels). In order to confirm that this observation is specific, we also stained neurons with an anti-N-terminus GluR1 antibody (Fig. 4, lower panels). It should be noted that, with the anti-N-terminus antibody, the percentage of intensely labeled cells in the bFGF-treated cultures did not differ from the control (63.9%±5.9 in control culture, 58.8%±4.8 in bFGF-treated cultures n=5; p=0.299). These observations suggest that the bFGF-dependent decrease in SAP97 protein might result in the exposure of the GluR1 C-terminal domain.

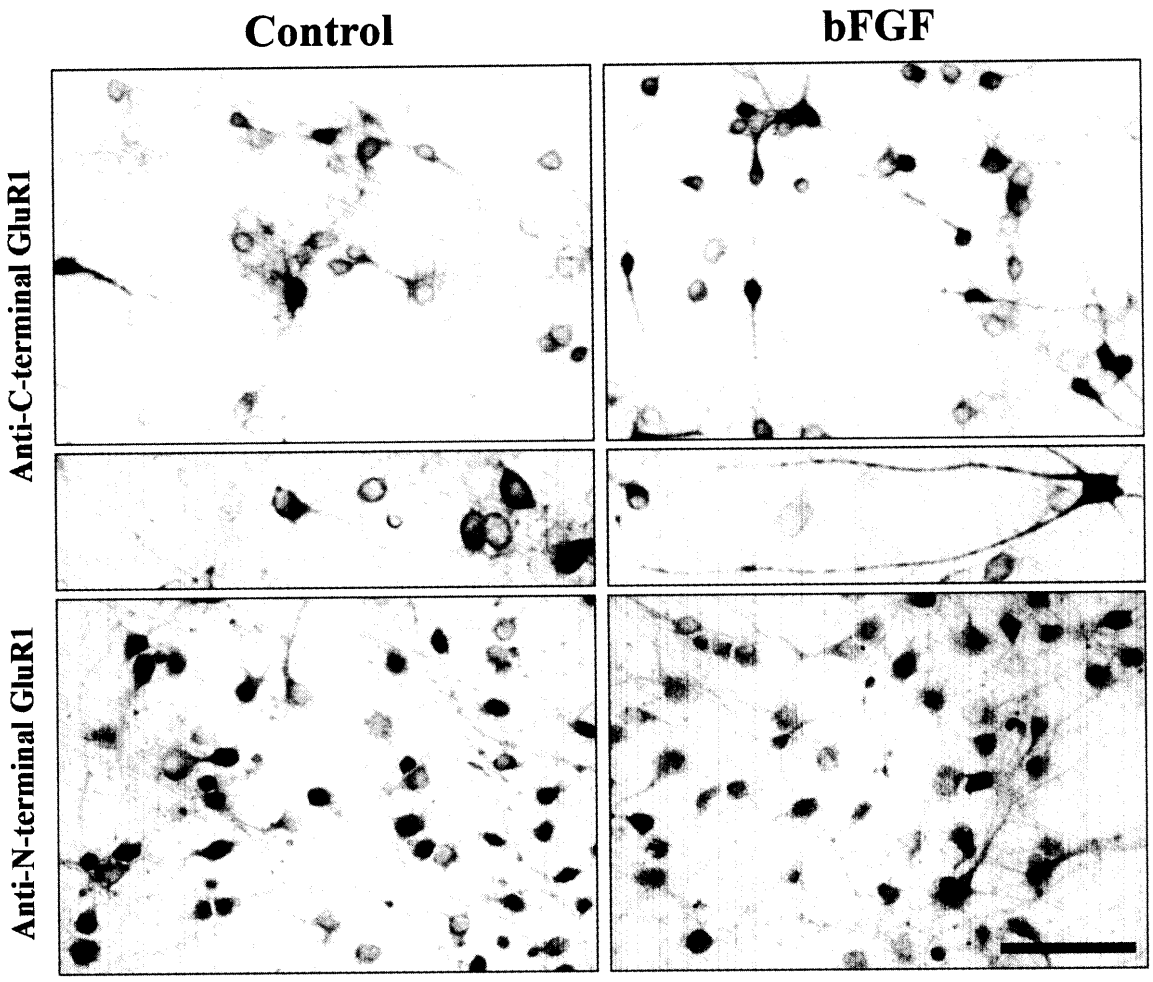

Fig. 4. Immunohistochemistry of the AMPA-type glutamate receptor subunit GluR1 in control (**A**) or bFGF-treated neuronal cultures (**B**). Neocortical neurons were dissociated and cultured in the absence or presence of bFGF (20 ng/ml; added daily) for 4 days, fixed with 4% paraformaldehyde in a phosphate buffered saline solution, and incubated with antibodies recognizing the C-terminus (upper and middle panels) or the N-terminus (lower panels) of GluR1. The results depict an increase in the number of strongly stained cells with the anti-C-terminus antibody without affecting the staining pattern with the anti-N-terminus antibody. Scale bar, 50 μm.

DISCUSSION

Specific members of the PDZ family of proteins have recently been implicated in important and distinct aspects of brain function and synaptic plasticity[16–18,20,22–25]. However, very little is known as to whether these molecules respond differently to various cytokines or growth factors. Resolving such an issue is important since these proteins have overlapping patterns of expression and interaction[23–26,38]. For instance, any factor that affects the expression of a particular PDZ protein might lead to a unique modulation of the cellular response to an afferent input, ultimately resulting in a divergence of the response at synapses that have different combinations of PDZ molecules.

The regulation of glutamatergic receptors by bFGF has received much attention as these receptors have been implicated in important aspects of brain physiology such as the induction of LTP or LTD[1,2,5,19,39,40]. Few reports discuss whether bFGF affects the AMPA-type receptors in neurons, their subcellular distribution, or the mechanisms applied

therein[2,29]. SAP97 and GRIP1 interact with the GluR1 and GluR2 subunits. They have also been implicated in the synaptic targeting and/or stabilization of AMPA receptors[18,20,21]. Such stabilization might increase neurotransmission and synaptic responsiveness to an incoming stimulus, whereas their removal could be crucial in avoiding the harmful effects of extended neuronal excitation that yields excitotoxicity and neuronal death[1,39,40]. To elucidate the effects of bFGF on such interactions, we used anti-N-terminal GluR1 or anti-N-terminal GluR2 antibodies to co-immunoprecipitate the interacting PDZ proteins (SAP97 and GRIP1, respectively) and compared their levels in both culture conditions. Our results show that bFGF down-regulated the PDZ proteins that interact with AMPA receptors (Fig. 1), which was reflected in the dissociation of GluR1 from its interaction with SAP97 (Fig. 3), rendering the GluR1 C-terminal epitope exposed to bind the corresponding antibody (Fig. 4). While elaborate studies have been published on the expression of PDZ proteins and glutamate receptors[29,30], most of the published data used 4% paraformaldehyde as a crosslinking reagent/fixative, and different, sometimes opposing, results have been obtained. Such has been the case for the C-termini of NMDA-type glutamate receptors and their interacting PDZ proteins PSD-95, PSD-93 and SAP102. This discrepancy has been resolved using pre-incubation with a protease or exposure to microwaves. Such denaturing procedures are known to reveal those epitopes that had been masked by interaction with partner molecules[30]. Labeling of AMPA receptors in young neurons in culture where "mature" synapses have not yet formed does not require such denaturating conditions[29,30]. Our staining results showed labeling in peri-nuclear and cytoplasmic compartments and in dendritic shafts, suggesting that by fixation time, significant amounts of the reactive molecules remained outside the postsynaptic compartment. Since bFGF did not affect the pattern of immunostaining with the anti-N-terminal GluR1 antibody nor the GluR1 protein levels, the bFGF-dependent increase in immunoreactivity with the C-terminal antibody is likely to represent a qualitative change in GluR1 antigenicity. In agreement, the reduced interaction between SAP97 and GluR1 C-termini in the bFGF-treated culture was presumably reflected in the unmasking of the C-terminal epitope. Our staining results also suggest that bFGF treatment might lead to the decreased surface expression of AMPA receptor proteins and possibly the amount of current contributed via the AMPA channels at the postsynaptic sites. Although our results do not exclude the possibility that bFGF might interfere with the translocation/targeting of PDZ proteins or various types of ionic channels to the synaptic compartment, the resolution of these issues requires more detailed investigations using electron microscopy as well as the study of the effects of bFGF on the electrophysiology of AMPA receptors.

bFGF can induce an opposite effect on a distinct member of the PDZ family of proteins. bFGF increased the expression of PSD-93, which interacts with the NMDA receptor subunits[40]. We cannot deny that bFGF might influence the NMDA receptor function in our neocortical cultures. Several previous studies using cerebellar granule cell cultures or hippocampal cultures, suggested that bFGF can indeed influence the expression and function of NMDA receptors[1,2,4-6,39,41]. It will therefore be interesting to study the consequences of our observations, namely whether PSD-93 contributes to the effects of bFGF on the NMDA receptor function in young neocortical cultures. Moreover, studying the role of PDZ proteins in neuronal cultures either incubated with bFGF or not, as well as their potential contribution to the survival promoting action of bFGF might also help in defining a new role for PDZ proteins. This will ultimately lead to the discovery of a new mechanism whereby the bFGF-dependent regulation of PDZ proteins provides a new protective mechanism against glutamate toxicity[4-8,14,39,41].

We have previously reported that the brain-derived neurotrophic factor enhances SAP97, GRIP1 and Pick1 protein expression[21]. Our current results further indicate that bFGF has an opposite effect on these same proteins, and ascribe a new role to bFGF in the regulation of PDZ protein expression. Together, these data suggest that cytokines and neurotrophins differentially regulate the expression of PDZ molecules potentially contributing to the synaptic diversity of glutamate receptor function. Future studies will help clarify the specific role of each PDZ protein in mediating the effects of bFGF on neuronal survival as well as synaptic development/plasticity.

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