Nuclear and Cytoplasmic Localization of Basic Fibroblast Growth Factor in Astrocytes and CA2 Hippocampal Neurons

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Fibroblast growth factors (FGFs) are known to stimulate mitogenesis in a variety of non-neuronal cell types and to support the survival *in vitro* of many neuronal cell types. The physiological role of FGFs in the CNS is currently not known. The present study determined the distribution in the rat CNS of a prominent member of the FGF family, basic FGF (bFGF). Immunohistochemical analysis showed that bFGF immunoreactivity was found predominantly in astrocytes throughout all regions of the CNS. In contrast, only a few neuronal populations were found to contain bFGF immunoreactivity, most prominent among them, neurons in the CA2 area of the hippocampus. This predominant localization of bFGF to astrocytes was confirmed by two other observations: (1) highly enriched cultures of astrocytes contained bFGF immunoreactivity and bioactivity, whereas highly enriched cultures of cerebral cortical neurons contained no detectable bFGF, and (2) neonatal rat cerebral cortex, which contains only a few differentiated astrocytes, also contained no detectable bFGF immunoreactivity and only low amounts of bFGF bioactivity. Immunocytochemical analysis also suggested that bFGF immunoreactivity was present in the nucleus as well as the cytoplasm of astrocytes and CA2 neurons. This nuclear localization was confirmed by EM analysis of the intracellular distribution of the immunoperoxidase reaction product. In addition, preparations of both nuclear and soluble fractions of brain extracts contained bFGF immunoreactivity and bioactivity. These data suggest that bFGF might be involved in mediating astrocytic influences on the late postnatal maturation and plasticity in the CNS, and that the nuclear localization of bFGF within astrocytes may play an important role in the differentiation of these cells. In addition, bFGF may play a similar role in a few specific neuronal populations, such as CA2 hippocampal neurons.

Fibroblast growth factors (FGFs) are members of the family of heparin-binding polypeptide growth factors that stimulate mitogenesis in a wide variety of cell types. The best-studied mem-

bers of the FGF family are acidic FGF (aFGF) and basic FGF (bFGF; see Burgess and Maciag, 1989, for review), but additional members of this family [HST (Taira et al., 1987), Int-2 (Moore et al., 1986), FGF-5 (Zhan et al., 1988), FGF-6 (Marics et al., 1989), and KGF (Marchese et al., 1990)] have been identified. The members in this family show structural homology and appear to share the properties of stimulating mitogenesis and binding to heparin with high affinity.

Recent observations suggest that FGFs may be of importance for the development and maintenance of nervous tissue. FGFs are present in relatively high levels in brain (Gospodarowicz et al., 1987; Burgess and Maciag, 1989), with aFGF and bFGF possibly being the main mitogenic factors extracted from adult brain (Thomas, 1987). Reported actions of FGFs *in vitro* include stimulation of mitogenesis in astrocytes (Pettmann et al., 1985), oligodendrocytes (Eccleston and Silberberg, 1985), and Schwann cells (Davis and Stroobant, 1990); promotion of fiber outgrowth in both PC-12 cells (Wagner and D'Amore, 1986) and adrenal chromaffin cells (Stemple et al., 1988); and promotion of survival or fiber outgrowth of neurons dissociated from cerebral cortex (Morrison et al., 1986), hippocampus (Walicke et al., 1986), retina (Lipton et al., 1988), cerebellum (Hatten et al., 1988), the septal area (Grothe et al., 1989), the ciliary ganglion (Schubert et al., 1987; Unsicker et al., 1987; Eckenstein et al., 1990), and sympathetic and sensory ganglia (Eckenstein et al., 1990).

The physiological relevance of these *in vitro* observations to the functions of aFGF and bFGF *in vivo* are not well understood for the following reasons. (1) Some of the *in vitro* effects of FGFs, such as the stimulation of mitogenesis of astrocytes, are not observed in the normal CNS, in spite of the fact that the CNS contains substantial levels of FGFs. (2) aFGF and bFGF may not be localized to an intracellular secretory pathway as they appear to lack a signal peptide sequence (Burgess and Maciag, 1989), a feature that is generally thought to be necessary for the efficient secretion of polypeptides (Walter and Lingappa, 1986); indeed, some fraction of total cellular FGF appears to be localized to the nucleus of cells that were transfected *in vitro* with FGF expression vectors (Renko et al., 1990). (3) The pleiotrophic effects of the FGFs observed *in vitro* seem somewhat nonspecific considering the very specific cellular interactions that guide CNS development and maintenance. This apparent discrepancy between the abundant FGF effects observed *in vitro* and the possibly more restricted role these factors may play *in vivo* could be explained by the hypothesis that FGFs become available to responsive cells *in vivo* only at restricted times and locations.

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As a first step toward testing this hypothesis, it is necessary to determine the patterns of expression of FGFs in the nervous system, as well as to identify the cell types expressing FGFs in this tissue and to investigate whether nuclear localization of FGFs may not only be observed in transfection systems but also in normal cells *in vivo*. We earlier demonstrated that aFGF and bFGF show a differential distribution in grossly dissected areas of the nervous system (Eckenstein et al., 1991a), supporting the hypothesis that specific FGFs may be expressed in restricted locations. The present study uses both light- and electron-microscopic immunohistochemical localization of bFGF, in combination with biochemical and bioassay methods, to determine the cellular and ultrastructural localization of bFGF in the rat CNS.

Some of the data reported here have been presented in short form at a recent conference (Eckenstein et al., 1991b).

Materials and Methods

Immunohistochemistry. Newborn and adult female Sprague-Dawley rats were anesthetized and perfused through the heart as follows: adults were first perfused with 50 ml of 100 mM sodium phosphate, pH 7.2, followed by 300 ml of 5% (v/v) formalin in the same buffer; newborns were perfused with 5 ml of 5% formalin in buffer only. Tissues were then dissected and postfixed in 5% buffered formalin for 1 hr at room temperature and sunk in 30% sucrose, 100 mM sodium phosphate, pH 7.2, and serial 50- μ m-thick frozen sections were prepared, spanning from olfactory bulb to cervical spinal cord.

Sections were first incubated for 2 hr in 100 mM Tris buffer, pH 7.8, containing 150 mM NaCl, 0.5% Triton X-100, 10% horse serum, and 0.05% sodium azide (Buffer A), followed by overnight incubation in one of the following primary antibodies diluted in Buffer A: mouse monoclonal anti-bFGF, ascites fluid 3386 (a generous gift from Dr. C. Hart, Zymogenetics) diluted 1:10,000; mouse monoclonal anti-glial fibrillary acidic protein (anti-GFAP) (Boehringer), 2 μ g/ml; or mouse control ascites (Sigma), diluted 1:2000. Sections were then washed and incubated for 1 hr in biotinylated anti-mouse antibodies (diluted 1:100 in Buffer A; Vector), washed, incubated for 1 hr in an avidin-peroxidase complex (Vector, prepared as suggested by the manufacturer), washed, and reacted for 10 min with diaminobenzidine (1 mg/ml) and 0.03% hydrogen peroxide in phosphate-buffered saline. The stained sections were mounted and coverslipped according to standard procedures. The mouse monoclonal antibody to bFGF was preadsorbed with 1 μ g/ml of human recombinant bFGF in experiments designed to ascertain the specificity of bFGF label observed.

For electron microscopic localization of bFGF immunoreactivity, tissue was prepared as described above, except that vibratome sections were used instead of frozen sections, and the concentration of Triton X-100 in Buffer A was reduced to 0.2%. Stained sections were then reacted for 1 hr with 1% osmium tetroxide in 100 mM cacodylate buffer, pH 7.3, and embedded in Embed 812/Araldite. Thin sections (ca. 300 Å thick) were cut and examined on a JEOL TEMSCAN transmission electron microscope. Cells were identified as astrocytes by the presence of glial filaments and rosettes of free ribosomes; neurons were identified by the presence of abundant cytoplasm with long segments of rough endoplasmic reticulum.

Double immunofluorescent localization of bFGF and GFAP was performed by simultaneously incubating sections in the mouse monoclonal antibody (see above) and a rabbit antiserum to GFAP (diluted 1:500; a gift of Dr. G. Nilaver), followed by incubation in fluorescein-labeled goat anti-mouse and rhodamine-labeled goat anti-rabbit antibodies (diluted 1:100; Cappel). Nuclei were labeled in these sections by incubation for 5 min in fluorescent Hoechst dye 33258 (1 μ g/ml). The resulting green, red, and blue immunofluorescent labels were analyzed in a Zeiss epifluorescence microscope using narrow-band filters. Control mouse ascites fluid and rabbit nonimmune serum were substituted for the primary antibodies in order to ascertain the specificity of staining observed.

Cultures of cortical astrocytes and neurons were immersion fixed in 5% buffered formalin and stained for bFGF, GFAP, and neurofilament (antibody RT 97, ascites, diluted 1:2000; Developmental Hybridoma

Bank; see Wood and Anderton, 1981) immunoreactivity using methods identical to those described above for the staining of tissue sections.

Preparation of tissue extracts and supernatants. Adult female Sprague-Dawley rats and newborn rat pups were killed by asphyxiation with carbon dioxide, and tissues were dissected, frozen immediately, and stored at -70°C for no longer than 14 d. Tissues were thawed and quickly homogenized in 10 ml/gm of ice-cold 20 mM Tris, pH 8.2, containing 600 mM NaCl. This concentration of NaCl was found in pilot experiments to be sufficient to extract all membrane-bound bFGF. The homogenates were centrifuged for 10 min at $15,000 \times g$, supernatants were collected, and bFGF was determined by Western blot analysis and bioassay as described below. Protein concentration in supernatants was determined using a Coomassie blue binding assay (Bio-Rad). Tissues from at least three different animals were assayed for all data presented in this study.

Preparation of nuclear pellets and supernatants. The forebrain from adult rats was homogenized in 10 ml/gm of cold 20 mM Tris, pH 8.2, containing 1% (w/v) CHAPS and 300 mM sucrose. The homogenate was passed through two layers of lens paper and centrifuged for 10 min at $800 \times g$. The supernatant was carefully removed from the pellet and dialyzed against 20 mM Tris, pH 8.2, containing 600 mM NaCl. The pellet was extracted with 2 M NaCl in 20 mM Tris, pH 8.2 (5 ml/gm starting tissue weight), and centrifuged for 20 min at $20,000 \times g$, and the supernatant was dialyzed as above. Protein (see above) and DNA concentrations (Labarca and Paigen, 1980) were quantified in these fractions, and the presence of bFGF was determined by Western blot analysis and bioassay as described below.

Preparation of tissue cultures. Astrocyte cultures were prepared in a manner similar to that described by McCarthy and deVellis (1980). Briefly, cerebral cortices from postnatal day 2 rat pups were removed, cleaned of meninges and blood vessels, and gently minced. The pieces of tissue were enzymatically dissociated by incubation in 0.2% (w/v) trypsin at 37°C for 25 min and then dispersed mechanically by trituration. Undissociated tissue and cell clumps were removed by passing the cell suspension through 130 μ m and 15 μ m Nitex filters. Cells were plated on tissue culture dishes (10 cm diameter, 10^7 cells per dish) in modified Leibowitz L-15 medium containing bicarbonate, 10% (v/v) fetal bovine serum, penicillin, streptomycin, glutamine, and 6 mg/ml glucose. Cells were grown close to confluence, and then the medium was changed to minimum essential medium (MEM), containing N2 supplement (Bottenstein and Sato, 1979), and the cultures were grown in this serum-free medium for an additional 7 d.

Neuronal cultures from postnatal rat cortex were prepared as previously described (Huettnner and Baughman, 1986). Cortical hemispheres from neonatal rat pups were cleaned of meninges and blood vessels, dissected, and dissociated by incubation in 20 U/ml papain at 37°C for 20 min, followed by gentle trituration. The cell suspension was passed through a 130 μ m Nitex filter to remove clumped cells, and the dissociated cells were plated on tissue culture dishes (10 cm diameter, 5×10^7 cells per dish) in MEM containing N2 supplement, 2×10^{-5} M fluorodeoxyuridine, 2×10^{-5} M uridine, and 10% MEM-based astrocyte-conditioned medium.

The purity of the different cell type-specific cultures was determined by immunohistochemical localization of GFAP and neurofilament immunoreactivity by the methods described above.

Confluent astrocyte and 6-d-old neuronal cultures were homogenized in 20 mM Tris, pH 7.2, containing 600 mM NaCl, 1% Chaps, 5 mM EDTA (1 ml per plate) and dialyzed against 20 mM Tris, pH 7.2, 600 mM NaCl. Western blot analysis and bioassay were used to determine bFGF in the dialysate as described below.

Bioassay. The total amount of mitogenic activity present in tissue and cell culture extracts was determined using a serum-free ^3H -thymidine incorporation assay described earlier (Shipley, 1986; Eckenstein et al., 1991a). Briefly, AKR-2B cells were transferred into 24-well culture plates (10,000 cells per well) in McCoy's 5A medium supplemented with 5% (v/v) fetal bovine serum and incubated for 5 d, by which time the cells formed a confluent monolayer. The cells were incubated for an additional 2 d in serum-free MCDB 402 medium (Shipley and Ham, 1981). Fresh MCDB 402, supplemented with varying concentrations of bFGF or cell or tissue extracts, was then added, and 22 hr later the cultures were pulsed with 1.0 μCi of ^3H -thymidine for 1 hr. The relative incorporation of ^3H -thymidine into cold 10% trichloroacetic acid-insoluble material was then determined. Total mitogenic activity in extracts was determined from dose-response curves for the extracts. One unit of mitogenic activity was defined as the amount that yields half-maximal stimulation per milliliter of assay medium.

Table 1. The number of bFGF-positive neurons and astrocytes in CA1 and CA2 of hippocampus

	Astrocytes		Neurons		Unidentified cells	
	bFGF positive	Unstained	bFGF positive	Unstained	bFGF positive	Unstained
CA1	44	7	6	195	15	40
CA2	75	5	51	123	5	12

The numbers of bFGF-positive and -negative neurons and astrocytes were counted in thin sections prepared from blocks of similar size from indicated areas of adult rat hippocampus. Cell types were classified as described in Materials and Methods. Note that, in good agreement with light microscopic observations, labeled neurons are mostly confined to area CA2. In addition, the vast majority of labeled neurons had irregularly shaped nuclei, whereas unstained neurons in CA2 had nuclei of more regular shape (see Fig. 3).

This assay detects not only bFGF, but also several other mitogenic growth factors (Shipley, 1986); therefore, the amount of bFGF in extracts was determined as the difference between total mitogenic activity and that remaining after indirect immunoprecipitation of bFGF from extracts by a monoclonal antibody (3386; same as used for immunohistochemistry). The immunoprecipitation procedure was as follows: 0.5 μ l of 3386 ascites and 500 μ l of sample were incubated for 2 hr, 10 μ l of rabbit anti-mouse antibodies (2 mg/ml) were added and incubated for 1 hr, and 100 μ l of *Staphylococcus aureus* cell walls (from Calbiochem) were added and incubated for 30 min. The mixture was centrifuged, and the amount of mitogenic activity remaining in the supernatants was quantified using the AKR-2B cell assay. Nonspecific immunoprecipitation controls were performed by substituting control ascites for 3386 ascites. All precipitation steps were carried out at 4°C.

Western blot analysis. Supernatants from cell and tissue extracts were prepared as described above, diluted with water to 300 mM NaCl, and applied onto small heparin-agarose columns (0.5 ml vol; from Bio-Rad). Next, the columns were washed with 20 mM Tris, pH 8.2, and then eluted with 0.5 ml of 0.4% sodium dodecyl sulfate (SDS) at 50°C. This protocol allows the roughly quantitative recovery of FGFs in the column eluates (Eckenstein et al., 1991a,b). The column eluates were concentrated to 50 μ l using a Speedvac apparatus. The eluates, and pure aFGF (a gift from Dr. K. Thomas, Merck) and bFGF (a gift from Dr. C. Hart, Zymogenetics) as standards, were separated electrophoretically in a 16% polyacrylamide gel in the presence of SDS following standard methods (Laemmli, 1970). Proteins in the gel were electroblotted onto nitrocellulose, followed by immunochemical detection of transferred bFGF. The detection protocol consisted of incubating the nitrocellulose with antibodies specific for bFGF (mouse monoclonal antibody 3386, 1:10,000 dilution of ascites), followed by incubation with biotinylated secondary antibodies and by a routine avidin/alkaline phosphatase staining procedure (from Bethesda Research Laboratories).

Results

Immunohistochemical analysis of bFGF distribution. We previously described a mouse monoclonal antibody that specifically recognized bFGF on Western blots (Eckenstein et al., 1991a). This same antibody was found to be useful for immunohistochemical localization of bFGF in 5% formalin-fixed adult rat CNS. Pilot studies revealed that fixatives stronger than 5% formalin, especially those containing glutaraldehyde (as little as 0.1% was tested), substantially reduced or abolished staining. Throughout adult rat CNS, bFGF immunoreactivity was found predominantly in small (<10 μ m) cells, some of which possessed

lightly stained processes (Fig. 1A,C). Double immunofluorescent localization of bFGF and GFAP demonstrated that these small cells were astrocytes (Fig. 2). Distinct bFGF immunoreactivity in neurons was found in only two areas of the CNS: pyramidal neurons in the CA2 region of hippocampus (Fig. 1E,F) and neurons in retrosplenial areas of the cerebral cortex (not shown). In contrast to the reproducibly strong bFGF immunoreactivity observed in astrocytes of adult cerebral cortex, no detectable bFGF immunoreactivity was found in the cortex of neonatal rats (Fig. 1A,B). Finally, bFGF immunoreactivity was also observed in non-neuronal cells lining the ventricles (not shown). All the bFGF staining described here was abolished by preincubation of the monoclonal antibody solution with 1 μ g/ml of recombinant bFGF (Fig. 1D).

The intracellular distribution of bFGF immunoreactivity in labeled cells had an uneven appearance, with lighter labeling of putative cytoplasmic areas and more intense labeling of putative nuclear areas (Figs. 1F, 2A). Thus, the cellular and ultrastructural distribution of bFGF immunoreactivity (as indicated by the peroxidase reaction product) in hippocampus was analyzed by electron microscopy. Confirming the light microscopic observations, bFGF immunoreactivity was found in astrocytes throughout the hippocampus and in neurons largely confined to the CA2 region (Table 1, Fig. 3). Interestingly, stained neurons in CA2 had a morphology somewhat different from unlabeled neurons in that same area (Fig. 3B), suggesting that a specific subpopulation of CA2 neurons contains bFGF immunoreactivity. Strong bFGF label was observed in the nuclei of both astrocytes and CA2 hippocampal neurons, and definitive staining was also seen in the cytoplasm of the labeled neurons (Fig. 3). The poor ultrastructural preservation of the tissue, caused by the weak fixation necessary to obtain acceptable bFGF staining intensity, did not allow the unambiguous demonstration of bFGF immunoreactivity in the cytoplasm of astrocytes. Nevertheless, since distinct labeling was observed of astrocytic processes in the light microscope (Figs. 1A,C; 2A), we conclude that bFGF immunoreactivity is present in both cytoplasmic and the nuclear compartments of astrocytes and CA2 hippocampal neurons.

Highly enriched cultures of cortical astrocytes and neurons

Figure 1. Localization of bFGF immunoreactivity in coronal sections of the rat CNS. bFGF immunoreactivity was localized using a monoclonal antibody to bFGF and an immunoperoxidase method. *A*, In adult neocortex, many small (>10 μ m diameter), evenly distributed cells with fine processes are stained. *B*, No specific staining is observed in neonatal neocortex. *C*, Numerous stained small-diameter cell bodies are also found in white matter, such as the external capsule. *D*, Preabsorption of the monoclonal antibody to bFGF with 1 μ g/ml of recombinant bFGF abolishes all specific staining. External capsule is shown, from a section adjacent to the one shown in *C*. *E*, Large neurons are stained in hippocampal area CA2 but not in adjacent hippocampus (arrow points to the center of area CA2). *F*, The labeled neurons in CA2 (arrowhead) are easily distinguished from the ubiquitous small cell bodies (arrow). Note that putative nuclear structures are more heavily stained than cytoplasmic areas of labeled cells. Scale bars: *B* (also for *A*), 18 μ m; *D* (also for *C*), 8 μ m; *E*, 120 μ m; *F*, 12 μ m.

Figure 2. Double immunofluorescent localization of bFGF and GFAP immunoreactivity, with simultaneous fluorescent localization of DNA in nuclei. A section through adult entorhinal cortex is shown, which was stained simultaneously with mouse monoclonal antibody to bFGF and a polyclonal rabbit antiserum to GFAP, followed by incubation with fluorescein-labeled anti-mouse and rhodamine-labeled anti-rabbit antibodies and Hoechst dye 33528 (to stain nuclei), and viewed with appropriate fluorescence filters (see Materials and Methods for details). *A*, bFGF immunoreactivity; *B*, GFAP immunoreactivity; *C*, Hoechst stain of nuclei. Note that bFGF and GFAP immunoreactivity is present in the same subpopulation of cells and that the bFGF immunoreactivity is strongest in nuclear regions of labeled cells, whereas GFAP immunoreactivity is largely confined to the processes of these same cells. Scale bar, 8 μ m.

were also analyzed for bFGF immunoreactivity. Cultures of cortical astrocytes were found to contain many bFGF-positive cells (Fig. 4*A,B*), although the number of bFGF-positive cells was somewhat lower than the number of GFAP-positive cells. In contrast, virtually all GFAP-positive cells *in vivo* appeared to contain bFGF immunoreactivity (Table 2). In addition, the bFGF staining of astrocytes in culture was largely restricted to the nucleus, where it was clearly most intense in what appeared to be the nucleolus; very little bFGF immunoreactivity was observed in the cytoplasm of cultured astrocytes. The differences in localization of bFGF immunoreactivity between astrocytes in culture and *in vivo* may be due to a number of reasons: limits of the sensitivity of the staining technique in culture, differences in fixation of cells in culture versus *in vivo*, and differences in the environment of cells grown *in vitro*. In good agreement with observations *in vivo*, cultures highly enriched in cortical neurons contained only a very few distinctly bFGF-positive cells (Fig. 4*C,D*), which were most likely to be an occasional astrocyte.

Analysis of bFGF activity. We sought to confirm the immunohistochemical localization of bFGF reported here by using an independent measure of the amount of bFGF present in the cells and tissues investigated. bFGF is a mitogen for AKR-2B cells in culture, and the total amount of mitogenic activity present in extracts can be determined by measuring the stimulation of 3 H-thymidine incorporation into the DNA of these cells (Shipley, 1986; Eckenstein et al., 1991a). This assay alone, however, cannot differentiate between bFGF and other mitogenic growth factors such as epidermal growth factor. Thus, the percentage of total mitogenic activity due to bFGF was determined by comparing total mitogenic activity with that remaining after bFGF had been removed from these extracts by indirect immunoprecipitation. The results of this assay confirmed all the results obtained by immunohistochemical localization of bFGF, in that (1) bFGF levels were highest in enriched cultures of cortical astrocytes and very low in cultures of cortical neurons; (2) significant levels of bFGF were observed in mature cerebral cortex, while levels were low in newborn cortex; and (3) both nuclear and soluble fractions of adult rat CNS contained bFGF (Table 2).

It is not surprising that pure cultures of astrocytes contained higher levels of bFGF per milligram of protein than mature cerebral cortex which contains many cell types, of which only astrocytes appear to contain detectable amounts of bFGF.

Since detergent (1% CHAPS) was used in the preparation of the nuclear fraction, the soluble fraction undoubtedly contained cytoplasmic as well as membrane-associated molecules. Extraction of tissues without detergent results in the solubilization of similar amounts of mitogenic activity (Eckenstein et al., 1991a); thus, the majority of bFGF in the solubilized fraction very likely is of cytoplasmic origin. The nuclear fraction contained more than 75% of the total DNA, as determined by a fluorometric assay, and the ratio of DNA to protein was greater than eightfold higher in the nuclear fraction than in the starting homogenate. Microscopic analysis confirmed this enrichment of nuclei in the nuclear fraction (Fig. 5).

Western blot analysis of bFGF. Recent reports indicate the existence of at least three different molecular weight forms of bFGF, possibly resulting from differential initiation of translation (Florkiewicz and Sommer, 1989; Renko et al., 1990; Bugler et al., 1991). Therefore, it was of interest to determine whether these forms were expressed in the cultured cells and tissues of rat CNS analyzed here. Western blot experiments,

Figure 3. EM localization of bFGF immunoreactivity in the CA2 area of adult hippocampus. *A*, Intense staining is found in the nucleus of an astrocyte, as identified by the presence of glial filaments. Astrocytic cytoplasm is difficult to observe, due to the mild fixation conditions used to preserve bFGF immunoreactivity. *B*, Strong staining is also seen in the nucleus (*N*) of a labeled CA2 neuron, but distinct staining can also be seen in the cytoplasm (*C*) of this cell. An adjacent neuron shows no staining in either the nucleus (*n*) or the cytoplasm (*c*); arrowheads delineate the cell boundary of the unstained neuron. Note the irregular shape of the nucleus in the stained neuron and the round, regular shape of the nucleus in the unstained neuron. This irregularity of nuclear shape was typical for the great majority of stained CA2 neurons, whereas the majority of unstained neurons showed more regular nuclear morphology. Scale bars: *A*, 3.5 μ m; *B*, 5 μ m.

Figure 4. Immunoperoxidase localization of GFAP, neurofilament, and bFGF in cultured cortical astrocytes and neurons. *A*, The vast majority of cells in the astrocyte culture are GFAP positive. *B*, Most, but not all, astrocytes in a sister culture contain bFGF immunoreactivity. The bFGF staining appears to be localized mostly to the nucleus and nucleolus of stained astrocytes. *C*, The majority of cells in neuronal cultures contain neurofilament immunoreactivity. *D*, Only very rarely is a bFGF-positive cell observed in neuronal sister cultures. This staining also appears mostly nuclear and is found exclusively in large cells with a morphology indicative of astrocytes that are present in low numbers in these neuronal cultures (see also Table 2). Scale bar, 8 μ m.

Figure 5. Fluorescent and phase microscopic inspection of nuclear preparations from adult forebrain. Nuclei were prepared and stained with Hoechst dye 33528 as described in Materials and Methods. *A*, Nuclei, identified by their strong fluorescence following dye binding, constitute the majority of material in such preparations. *B*, Additional contaminating nonfluorescent material, however, is detected by phase-contrast microscopy. Scale bar, 12 μ m.

using the same monoclonal antibody to bFGF employed for immunohistochemistry, were performed to investigate this possibility (Fig. 6). Adult rat cerebral forebrain and cultured astrocytes expressed all three forms of bFGF (18 kDa, 21.5 kDa, and 22.5 kDa in molecular mass) in similar abundance. All three forms were also present in soluble fractions prepared from adult rat CNS, whereas nuclear fractions contained nearly exclusively the 21.5 kDa and 22.5 kDa forms. In agreement with results described above, cultured cortical neurons did not contain detectable amounts of any of these forms of bFGF.

Discussion

As a step toward better understanding the physiological function of FGFs in the nervous system, we investigated the cellular and intracellular distribution of bFGF in the rat CNS. We found that bFGF immunoreactivity was present primarily in astrocytes throughout the CNS as well as in a few identified neuronal

Figure 6. Specific detection of bFGF by Western blot. Different cell and tissue extracts were prepurified using heparin-Affigel and processed for Western blot detection of bFGF immunoreactivity as described in Materials and Methods. Three bands of slightly different molecular mass (18 kDa, 21.5 kDa, and 22.5 kDa) of bFGF immunoreactivity are seen in extracts prepared from cultured astrocytes (lane 1, 2 mg starting protein), but no detectable bFGF bands are present in extracts from cultured neurons (lane 2, 4 mg starting protein). Nuclear preparations from forebrain contain only the two higher molecular weight forms of bFGF immunoreactivity (lane 3, 2 mg starting protein), whereas soluble fractions from forebrain contain all three forms (lane 4, 10 mg starting protein). The specificity of the monoclonal antibody to bFGF used is demonstrated by the fact that 200 ng of human recombinant aFGF (lane 5) are not stained, whereas 1 ng of human recombinant 18 kDa bFGF results in a strongly labeled band (lane 6). Numbers on the left indicate molecular weight.

populations, including pyramidal neurons in the CA2 region of hippocampus and neurons in the retrosplenial cortex. That bFGF is localized in adult CNS mainly to astrocytes was confirmed by two other observations: (1) highly enriched cultures of astrocytes, confirming an earlier report (Ferrara et al., 1988; these authors, in addition, demonstrated the expression of bFGF mRNA in cultured astrocytes), contained high levels of bFGF immunoreactivity and bioactivity, whereas highly enriched cultures of cerebral cortical neurons contained no detectable bFGF; and (2) neonatal rat cerebral cortex, which contains few differentiated astrocytes, also had no detectable bFGF immunoreactivity and only low amounts of mitogenic activity. Interestingly, bFGF immunoreactivity was found in the nucleus as well as in the cytoplasm of labeled cells.

These results are in conflict with previous immunohistochemical studies of the distribution of bFGF that had indicated a much more widespread neuronal localization of bFGF (Pettmann et al., 1986; Finklestein et al., 1988; Janet et al., 1988). Our results, however, are in good agreement with a recent report describing the distribution of bFGF mRNA by *in situ* hybridization, including the demonstration of specific expression of bFGF mRNA by hippocampal CA2 neurons (Emoto et al., 1989). Moreover, the present observations are consistent with our earlier findings that bFGF bioactivity is distributed relatively evenly throughout the CNS and is present in stumps of transected optic nerve that lack axons and hence contain only glia (Eckenstein et al., 1991a). The differences in immunohistochemical localization of bFGF in the nervous system described here and those reported previously may be due to differences in the source of antibodies; the present study used a monoclonal antibody to bFGF that appears to be monospecific for bFGF (Eckenstein et al., 1991a), whereas previous studies reporting widespread localization of bFGF to all neurons in CNS utilized polyclonal antibodies that, because of the significant sequence homology among the seven members of the FGF family, may have cross-reacted with FGFs not recognized by the monoclonal antibody

Table 2. Levels of bFGF bioactivity in tissues and cell cultures

	Mitogenic units/mg protein + control antibody	Mitogenic units/mg protein + antibody to bFGF	Mitogenic units of bFGF per mg protein	Number of GFAP- positive cells	Number of bFGF- positive cells
Adult cerebral cortex	27.5	10.5	16.0	43.7	46.1
Newborn cerebral cortex	3.3	<2.0	<3.3	1.1	0
Nuclear extract from adult forebrain	89.3	33.3	56.0	ND	ND
Soluble extract from adult forebrain	23.8	8.2	15.6	ND	ND
Cortical astrocytes <i>in vitro</i>	51.9	3.4	48.5	39.4	28.8
Cortical neurons <i>in vitro</i>	<2.0	<2.0	<2.0	3.8	1.2
bFGF (100 ng/mg of carrier protein)	126.6	<2.0	124.6		

The amount of bFGF activity was quantified by determining the amount of mitogenic activity precipitated by a monoclonal antibody to bFGF (see Materials and Methods for details; SE did not exceed $\pm 20\%$ of total values). The number of GFAP- and bFGF-positive cells is the mean number of stained cells observed in 10 fields of view of equal size (SE did not exceed $\pm 20\%$ of total values). Note that the levels of bFGF activity correlate well with the presence of GFAP-positive astrocytes, and that bFGF-activity is present in both soluble and nuclear extracts of forebrain. ND, Not determined.

used here. These other members of the FGF family may be more highly concentrated in neurons than bFGF. This would be consistent with our previously published results suggesting that aFGF is found in some classes of peripheral neurons (Eckenstein et al., 1991a,b). On the other hand, if these other members of the FGF family are mitogens like bFGF, then we should not have found such an excellent correlation of mitogenic activity with bFGF immunoreactivity in the CNS. Perhaps these other FGFs had no effect on the AKR-2B cells used for our biological assay, where aFGF, for example, is inactive in the absence of heparin (Eckenstein et al., 1991a). Clearly, additional studies with probes for other FGFs are needed to describe fully the distribution of FGFs in the CNS.

In general, polypeptide growth factors are expected to be made available to the cells that respond to them by secretion; thus, their intracellular distribution would be expected to be within the rough endoplasmic reticulum, the Golgi apparatus, and secretory granules (Walter and Lingappa, 1986). We have found that bFGF in astrocytes and CA2 neurons differed from this expectation in that significant amounts of bFGF immunoreactivity and bioactivity were present both in the nucleus and in the cytoplasm. Three bands of bFGF immunoreactivity of slightly different molecular masses (18 kDa, 21.5 kDa, and 22.5 kDa) were identified in Western blots of soluble fractions prepared from CNS, whereas only the two higher molecular mass bands were present in nuclear fractions. These observations agree well with earlier studies demonstrating a similar pattern of distribution of the different forms of bFGF in the nucleus versus the cytoplasm in cells that were transfected *in vitro* with bFGF expression DNA vectors (Renko et al., 1990). It should be noted that all three forms of bFGF appear to have similar mitogenic activity when added to fibroblasts in culture (Florkiewicz and Sommer, 1989).

Since bFGF lacks the traditional hydrophobic signal peptide sequence (Burgess and Maciag, 1989) that appears to be needed to target polypeptides into the secretory pathway (Walter and Lingappa, 1986), our observation of intracellular localization of bFGF was not unexpected. This result appears to conflict with

a previous report that at least a small amount of bFGF may be present on the surface of cultured astrocytes because fiber outgrowth of cerebellar granule cells on astrocytes *in vitro* has been reported to be blocked by the addition to the culture medium of antibodies of bFGF (Hatten et al., 1988). This extracellular bFGF, however, may have been the result of cell lysis or release by an unconventional mechanism such as intracellular binding of bFGF to carrier proteoglycans that may subsequently be released (Gordon et al., 1989). Our own preliminary results indicate that although astrocytes *in vitro* contain significant stores of bFGF, little bFGF is present in medium conditioned by astrocytes, supporting the notion that bFGF is not efficiently released from intact cells.

The nuclear localization of bFGF was somewhat unexpected, but recent studies have shown that aFGF possesses a nuclear translocation sequence (Imamura et al., 1990). Although the nuclear translocation sequence of aFGF is not essential for the binding to cell surface FGF receptors, it is necessary for the mitogenic effect (Imamura et al., 1990). Observations that bFGF appears to be localized to the nucleus and especially the nucleolus at specific stages of the cell cycle (Baldin et al., 1990) might explain why in the present study the vast majority of astrocytes *in vivo* (which are likely to be arrested at the same stage of the cell cycle) contained bFGF immunoreactivity in the nucleus, whereas only about 70% of astrocytes *in vitro* (which are dividing and not synchronized in their cell cycle) contained nuclear bFGF. A recent study suggests that the presence of bFGF in the nucleus increases the synthesis of mRNA coding for specific ribosomal proteins (Amalric, 1991), and it may be of interest in this respect that specific inhibition of bFGF synthesis by bFGF antisense oligonucleotides in cultured glioma cells significantly reduced the rate of growth of these cells (Morrison, 1991).

These data, taken together with observations that FGF receptors are expressed widely throughout the nervous system (Heuer et al., 1990; Wanaka et al., 1990), allow the formulation of a working hypothesis as to the possible functions of bFGF in the CNS; that is, an important role for bFGF may be to mediate interactions of astrocytes with their environment in

order to regulate maturation and maintenance of the CNS. This overall function can be subdivided into distinct intra- versus intercellular functions of bFGF. Intracellular functions may include regulating the growth and differentiation of astrocytes and CA2 and retrosplenial neurons by a mechanism that does not involve release but instead involves translocation of newly synthesized bFGF to the nucleus. Possible intercellular functions of bFGF, such as promotion of mitogenesis in a variety of non-neuronal cell types and neurotrophic effects in a variety of neuronal cell types, may be mediated by bFGF released from astrocytes and acting extracellularly by binding to FGF receptors on the plasma membrane (Hatten et al., 1988). This extracellular bFGF may include low levels of bFGF released by unconventional mechanisms in normal CNS tissue, and larger amounts of bFGF released from intracellular stores during cell lysis after injury. Interestingly, the intracellular translocation of the FGF signal after binding to the surface receptor may also involve transport to the nucleus (Baldin et al., 1990). Thus, the intra- and intercellular functions of FGFs may involve similar pathways in the nucleus. Clearly, future studies are needed to test the prediction of two separate signaling pathways for FGF proposed by this working hypothesis.

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