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Internalization and Processing of Basic Fibroblast Growth Factor by Neurons and Astrocytes

Patricia A. Walicke[1] and Andrew Baird[2]

[1]The Department of Neuroscience, University of California at San Diego, La Jolla, California 92037 and [2]Department of Molecular and Cellular Growth Biology, The Whittier Institute for Diabetes and Endocrinology, La Jolla, California 92037

The fate of iodinated basic fibroblast growth factor (FGF) after its binding to cultured astrocytes and hippocampal neurons was studied. Autoradiography after light and electron microscopy establishes that, if cells are returned to 37°C, the ^{125}I–basic FGF bound internalizes into vesicles in the cytoplasm, localizes to the perinuclear cytoplasm, and is translocated to chromatin structures of the nucleus. The radiolabeled protein is long-lived, a finding confirmed by biochemical analyses. Polyacrylamide gel electrophoresis and autoradiography of both hippocampal neurons and astrocyte extracts reveal that these cells internalize ^{125}I–basic FGF and then metabolize it to three major heparin-binding peptides with molecular weights of 15.5, 9, and 4 kDa. These peptides are initially detected 16 hr after binding to neurons and 4 hr after binding to astrocytes but are still detectable 48 and 16 hr, respectively, after initial binding (though present at lower levels). Immunoprecipitation with sequence-specific antisera to basic FGF reveals that the 15.5-kDa fragment is generated by cleavage at the carboxyl terminus, that the 9-kDa peptide contains the sequences between residues 30 and 87, and the 4-kDa peptide is a C-terminus fragment containing the sequence of basic FGF(106–120) but without basic FGF(139–146) immunoreactivity.

The internalization of basic FGF is required for this processing; the treatment of cells with trypsin and 2 M NaCl at different times after binding can only prevent the metabolism of basic FGF if it is performed immediately after binding. Similarly, WGA, which inhibits basic FGF binding to its high-affinity receptor, prevents the metabolism of basic FGF. The possible significance of a metabolic pathway that is responsible for the processing of basic FGF after its internalization by cells in the CNS is discussed in light of its potential function as a neurotrophic factor.

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Correspondence should be addressed to Andrew Baird, Ph.D., Department of Molecular and Cellular Growth Biology, The Whittier Institute for Diabetes and Endocrinology, 9894 Genesee Avenue, La Jolla, CA 92037.

Peptide growth factors are internalized by cells after binding to their cell-surface receptors (Schwab, 1977; Heldin et al., 1982; Rosenfeld et al., 1984; Bergeron et al., 1985; Carpenter, 1987; Levi-Montalcini, 1987). In most cell types, the internalized peptide is shifted into lysosomes and degraded. In neurons, however, internalization is often followed by retrograde or anterograde axonal transport, an event that is implicated in the mechanism of action of neurotrophic factors (Thoenen and Edgar, 1985; Levi-Montalcini, 1987) like basic fibroblast growth factor (FGF). Basic FGF supports the survival and differentiation of neurons from many regions of the PNS and CNS *in vitro* (Morrison et al., 1986, 1988; Walicke et al., 1986; Unsicker et al., 1987; Hatten et al., 1988; Walicke, 1988) and can prevent neuronal death *in vivo* (Anderson et al., 1988; Otto and Unsicker, 1990). Although its mechanism of action is not known, specific binding sites for basic FGF have been characterized in cultures of fetal hippocampal neurons (Walicke et al., 1989). Extracellular glycosaminoglycans (GAGs), particularly heparan sulfate proteoglycans, account for 40–70% of total nonspecific binding, but there also exists a high-affinity glycoprotein receptor on the cell surface of neurons (Walicke et al., 1989). Thus, there most likely exist specific receptor-mediated internalization, metabolism, and potentially transport of basic FGF by neurons. Indeed, the recent studies of Ferguson et al. (1990) established that basic FGF is transported anterogradely by retinal ganglion cells *in vivo*.

Binding studies on mesenchymal cell types have suggested that the basic FGF receptor is down regulated (Neufeld and Gospodarowicz, 1985; Olwin and Hauschka, 1986; Moscatelli, 1987). Unlike the case for epidermal growth factor (EGF), platelet-derived growth factor (PDGF), and insulin (Heldin et al., 1982; Mock and Niswender, 1983; Dunn and Hubbard, 1984; Bergeron et al., 1985; Carpenter, 1987), however, free ^{125}I-tyrosine does not rapidly appear in the medium after binding ^{125}I–basic FGF. Instead, basic FGF appears to be degraded into a set of smaller peptides that remain associated with cells for several hours (Moenner et al., 1987, 1989; Moscatelli, 1988). In a recent series of studies, we have characterized the receptor for basic FGF on mesenchymal cells (Feige and Baird, 1988) and cells in the CNS (Walicke et al., 1989) and found that there were significant differences. In the studies reported here, we have extended these studies to compare the fate of basic FGF after its binding to neurons and astrocytes. In an effort to determine whether the metabolism of basic FGF might mediate its cellular (Magnaldo et al., 1986; Coughlin et al., 1988) and proposed nuclear (Baldin et al., 1990; Renko et al., 1990) effects, immunoprecipitation studies were performed to identify the metabolic fragments.

Materials and Methods

Preparation of ^{125}I–basic FGF. Recombinant human basic FGF (Barr et al., 1988) was iodinated by the lactoperoxidase method and purified by heparin-Sepharose affinity chromatography as previously described (Baird et al., 1988). The specific activity averaged 105,000 cpm/ng. In all of the studies reported here, we have maintained the amino acid numbering system outlined by Esch et al. (1985) for the 146–amino acid protein.

Tissue culture. Hippocampi were removed from 18-d fetal Sprague–Dawley rats, dissociated with 0.25% trypsin and trituration, and grown in serum-free medium as previously described (Walicke et al., 1986; Walicke, 1988) Astrocyte cultures were established from cerebral cortex of 1–3-d-old rat pups as described (Walicke and Baird, 1988) and grown in 10% fetal calf serum in Dulbecco's modified Eagle's medium (DMEM). Cells were grown at a density of 10^6 cells/dish in 35-mm tissue culture dishes coated with polyornithine and laminin.

Radioreceptor assays. The medium used in the binding assays was identical to the respective serum-free formulations (Walicke, 1988) used to grow either neurons or astrocytes except for further addition of 20 mM HEPES (pH, 7.4) and 0.2% gelatin. The binding assays were performed essentially as described by Moscatelli (1987) as modified for neurons (Walicke et al., 1989). ^{125}I–basic FGF (1 ng/ml, 100,000 cpm/ng) was added to the cells, and they were incubated for 3–4 hr at 4°C. The cells were then rinsed three times with 0.2% gelatin in DMEM containing 20 mM HEPES (pH, 7.4). At this point, some cells were processed immediately, while others received fresh growth medium and were returned to the 37°C incubator for the indicated times.

Light microscopy and autoradiography. Cells for light microscopic autoradiography were grown on glass microscope slides at a density of $3 \times 10^4/cm^2$. The slides were coated with 0.5% gelatin, air dried, treated with 0.1 mg/ml polyornithine for 30 min, and then incubated overnight with 1 μg/ml laminin. For autoradiography, astrocytes were harvested with 0.25% trypsin, replated, and maintained for 2 d in serum-free medium.

After rinsing, cells were fixed with 4% glutaraldehyde in phosphate-buffered saline for 15 min and rinsed three times with DMEM plus 0.2% gelatin. They were then washed with water, dehydrated through a graded series of ethanol, defatted for 1 hr in 1:1 chloroform/methanol, rehydrated, and allowed to dry overnight. The slides were dipped in a 50% solution of Kodak NTB-2 emulsion, packaged with lead inserts between each sample, and exposed for 3–5 d at 4°C. The emulsion was developed with Kodak D19 and fixed with Rapid Fix. After thorough rinsing, the cells were stained with a 15-min incubation in 1% cresyl violet.

Electron microscopy and autoradiography. Cells were fixed by incubation with 2% glutaraldehyde in DMEM for 1 hr, rinsed three times with DMEM plus 0.2% gelatin and then with 0.125 M sodium phosphate, postfixed for 30 min in 1.3% osmium tetroxide, and dehydrated through a graded series of ethanol. The dish was filled with Polybed 812 (Polysciences), and the embedded neurons were peeled off the surface of the dish. The disks of Polybed were dipped in Kodak NTB-2 emulsion, exposed for 5 d at 4°C, and developed for light microscopic autoradiography as above. Heavily labeled cells were marked with a stylus, and then the emulsion was washed from the disk with dilute NaOH. The blocks were trimmed down to the cells of interest, and 100-nm sections were cut on a Reichardt OMU3 ultramicrotome and then mounted on Formvar-coated 300-mesh grids.

The grids were mounted on glass coverslips with Scotch tape. Monolayers of Ilford L-4 emulsion were formed with a nichrome-wire loop and applied to grids. The coverslips were packaged with lead inserts between samples and exposed for 1–2 months at 4°C. They were developed with Kodak D-19 (2 min, 18°C), followed by 1% acetic acid for 30 sec and 30% sodium thiosulfate for 3 min. The samples were stained by a 10-min incubation with a saturated solution of uranyl acetate in 50% ethanol, followed by 0.4% lead citrate in 0.1 M NaOH for 5 min. After samples were rinsed with 0.02 M NaOH and water, they were dried. The samples were examined using a Zeiss 109 electron microscope.

Metabolism of ^{125}I–basic FGF neurons and glia. At the indicated times, cells were washed with culture medium, scraped from the plates in solution A (0.15 M NaCl and 10 mM Tris, pH 7.4, containing 5 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 0.015 mg/ml pepstatin A, 0.1 mg/ml leupeptin, and 0.1 mg/ml aprotonin). The cells were collected by centrifugation for 10 min (14,000 × *g*, 4°C), and the pellet was solubilized in Laemmli's (1970) sample buffer.

Some experiments used heparin-Sepharose affinity chromatography to concentrate ^{125}I–basic FGF and metabolites from cell extracts. In these instances, cells were collected and sonicated in solution A plus 1.85 M NaCl. Unsolubilized debris was removed by centrifugation (14,000 × *g*, 10 min, 4°C). The extract was diluted to 0.15 M NaCl and applied to a column of heparin-Sepharose (gel vol, 0.25–0.5 ml). The proteins were eluted with step gradients of NaCl at 0.15, 0.6, 1.0, and 2.0 M NaCl in 10 mM Tris buffer (pH, 7.4). The eluates were dialyzed overnight against distilled water in dialysis tubing with a 3-kDa molecular-weight cutoff (Spectra/Por), lyophilized, and resuspended in Laemmli's sample buffer (Laemmli, 1970). Samples were chromatographed on 15% acrylamide gels prior to autoradiography.

Immunoprecipitation. The antisera to basic FGF were prepared in rabbits by immunizing animals with the indicated synthetic peptides conjugated to bovine serum albumin, ovalbumin, or keyhole limpet hemocyanin as previously described (Baird et al., 1985). The peptide sequences correspond to those of Esch et al. (1985) for basic FGF[1–146], and antibodies were generated against basic FGF[1–24]NH_2, [Tyr^{50}]-FGF[30–50]NH_2, [Tyr^{87}]-FGF[69–87]NH_2, FGF[106–120]NH_2, and FGF[139–146]OH. After lyophilization, the proteins eluted from the heparin-Sepharose column were resuspended in 400 μl RIPA buffer consisting of 0.01 M sodium phosphate (pH, 7.2), 0.15 M NaCl, 0.01% aprotonin, 1% NP40, 1.0% sodium deoxycholate, 0.1% SDS, 2 mM EDTA, and 50 mM NaF. The mixture was incubated for 1 hr at 4°C with 50 μl of protein A agarose. The agarose gel was removed by centrifugation, and 5 μl of antiserum was added to the supernatants. After incubation for 1 hr at 4°C, 50 μl of the protein A agarose was added, and the samples were incubated overnight at 4°C with agitation. The following day, the agarose beads were washed three times with RIPA buffer and once with 0.1% SDS in 10 mM Tris and 0.15 M NaCl. The samples were prepared for SDS-PAGE by boiling for 3 min in Laemmli's sample buffer (Laemmli, 1970).

Results

Basic FGF binding to neurons and astrocytes. In all of the experiments described here, ^{125}I–basic FGF (1 ng/ml, ~100,000 cpm/ng) was preincubated with hippocampal neurons or astrocytes for 2–3 hr at 4°C in order for binding to reach a steady state (Walicke et al., 1989). The fate of basic FGF bound by either neurons or astrocytes was first investigated using light microscopic autoradiography. When cells were fixed immediately after binding (Fig. 1*A,B*), label was distributed rather diffusely around the margins of the cells. For the neurons (Fig. 1*A*), grains could be seen outlining the soma and extending away from the cell in linear arrays (arrowheads). The cresyl violet counterstain reveals only nuclei and large proximal processes. The linear arrays show ^{125}I–basic FGF bound to finer neuritic processes, an interpretation supported by the observation of autoradiographic label associated with the fine processes detected by electron microscopic autoradiography (e.g., Fig. 2*D*). In the astrocyte cultures, label was distributed over the entire surface of the cells, but tended to be a bit denser along the cell margins at points of contact with the growth surface (Fig. 1*B*). Labeling with ^{125}I–basic FGF was specific and could be blocked with excess cold basic FGF but not by insulin, EGF, NGF, or transferrin (data not shown).

If cells are returned to 37°C for 16 hr after the binding assay, the distribution of label changes considerably. For neurons (Fig. 1*C*), the label along the processes disappears. Instead, there is a dense accumulation of grains over the cell somata. Under higher magnification (Fig. 1*E*), most of the label appears to lie over the perinuclear cytoplasm; however, a few grains appeared to be over the nucleus itself. A similar change is also seen for the astrocytes (Fig. 1*D,F*); the label appears in the cytoplasm adjoining the nucleus. Although the pattern is similar, the rate of ^{125}I–basic FGF redistribution observed by light microscopy is different between astrocytes and neurons. While neurons take

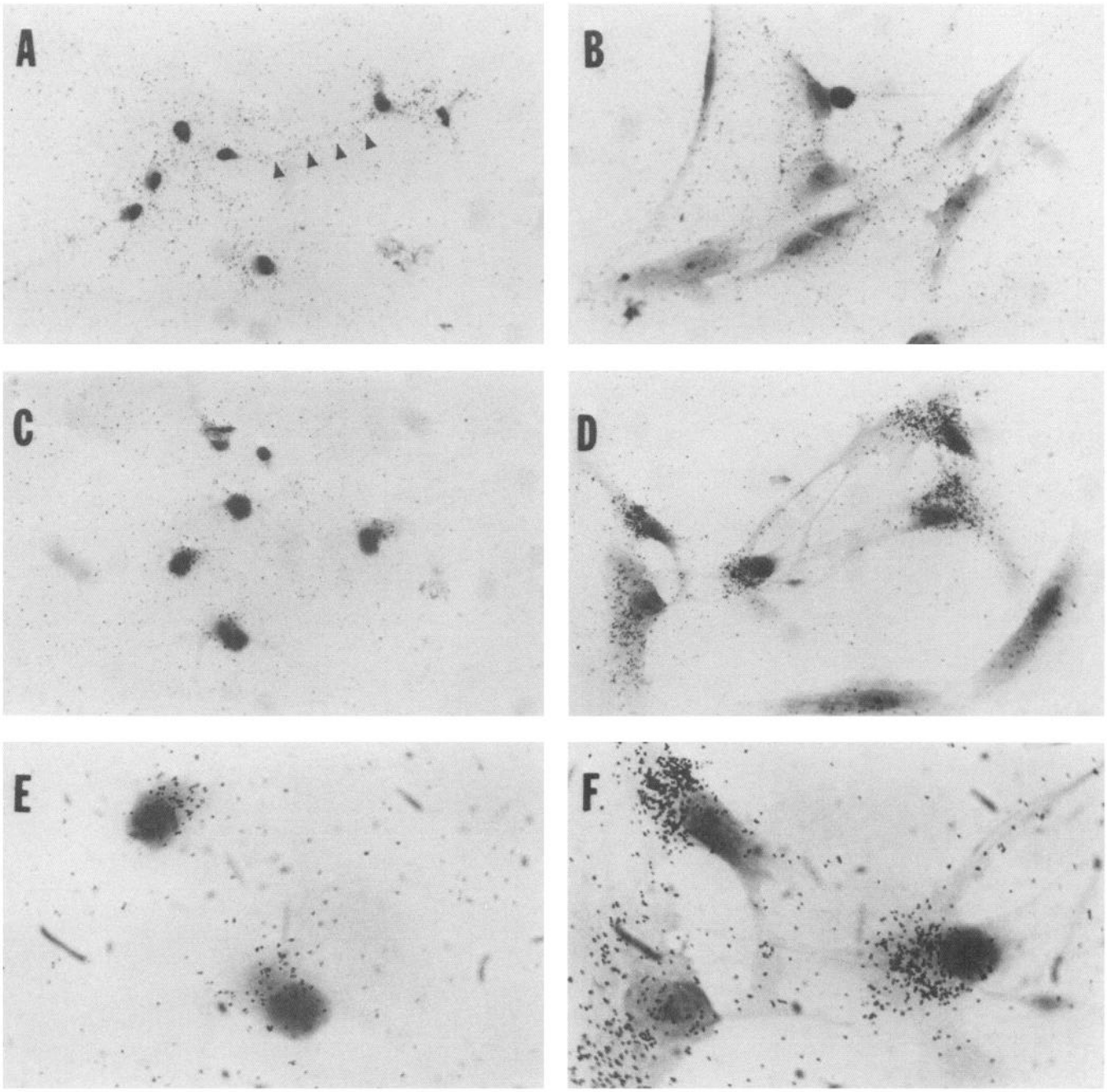

Figure 1. Light microscopic autoradiography of neuronal and astrocytic cultures. The binding of ^{125}I–basic FGF and autoradiography to hippocampal neurons (*A, C,* and *E*) and astrocytes (*B, D,* and *F*) was performed as described in the text. The cells were examined immediately after binding at 4°C (*A, B*) or after 16 hr at 37°C. *Arrowheads* in *A* show the linear array of label associated with neuronal processes. *E* and *F* are higher-magnification views of cells treated like those in *C* and *D*. Magnification: *A–D*, 38×; *E* and *F*, 95×.

16 hr at 37°C to show changes in distribution (see Fig. 1), astrocytes all show a much more rapid translocation often within 0.5–1 hr (not shown).

The distribution of label observed under light microscopy suggests internalization of ^{125}I–basic FGF by cells. However, whole-cell preparations cannot provide unequivocal localization. For example, the binding sites for ^{125}I–basic FGF may remain membrane associated and concentrated by lateral diffusion. Therefore, electron microscopic autoradiography was performed for more accurate subcellular localization. Astrocytes and hippocampal neurons were examined for internalization of ^{125}I–basic FGF (1 ng/ml, ~100,000 cpm/ng) after incubation for 4 or 16 hr at 37°C. The qualitative results obtained by electron microscopy at these time points were indistinguishable. An example of an astrocyte is given in Figure 2*A*. Several grains can be seen lying over vesicular structures in the cytoplasm. These are some of the most frequently labeled structures at this time point. At higher magnification, the astrocyte cytoplasm can be seen to contain several structures that concentrate label. The most abundant are with the endosomal apparatus and lysosomes (Fig. 2*B*). The second most frequent localization was overlying cytoplasm, in the vicinity of vesicles and ribosomes. Unex-

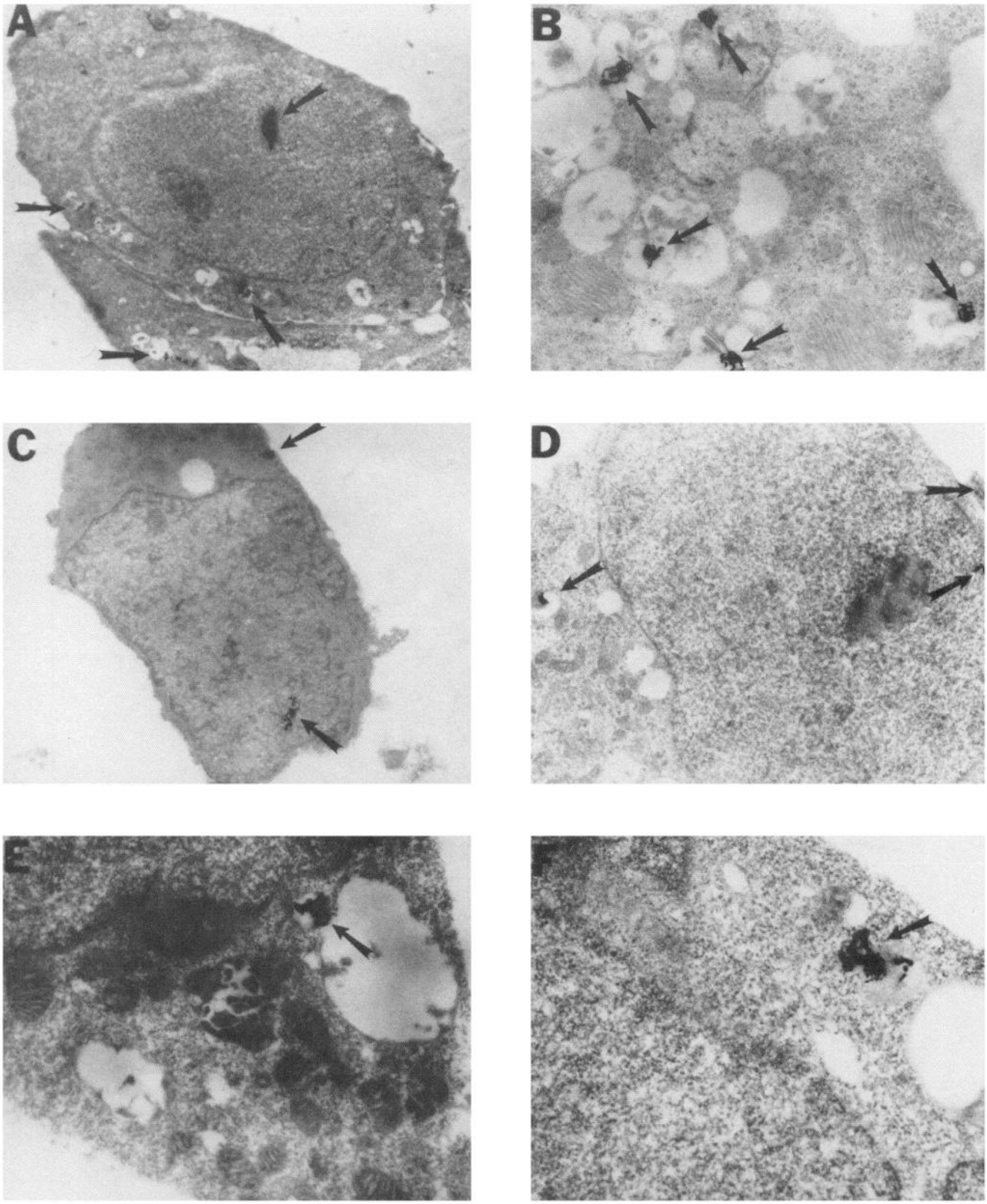

Figure 2. Electron microscopy of neuronal and astrocytic cultures after internalization of ^{125}I–basic FGF. Binding and autoradiography methods are given in Materials and Methods. Autoradiograms were exposed for 1 month except for *A*, which was exposed for two months. *Arrows* point to autoradiographic grains. *A* and *B* are astrocytes; *C–F* are neurons. Magnification: *A*, 2850×; *B* and *E*, 11,400×; *C*, 3800×; *D*, 6650×; *F*, 19,000×.

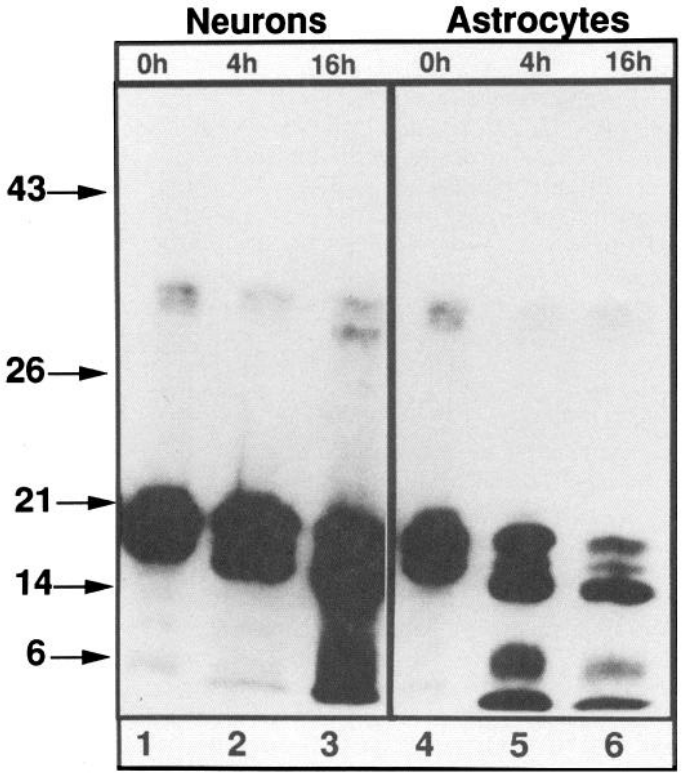

Figure 3. Metabolism of ^{125}I–basic FGF by neurons and astrocytes. Hippocampal neurons (*lanes 1–3*) or astrocytes (*lanes 4–6*) were incubated with ^{125}I–basic FGF and extracted immediately after the binding assay (*lanes 1, 4*), 4 hr after being returned to 37°C (*lanes 2, 5*), or 16 hr at 37°C (*lanes 3, 6*). Molecular weights (in kDa) are indicated to the *left*.

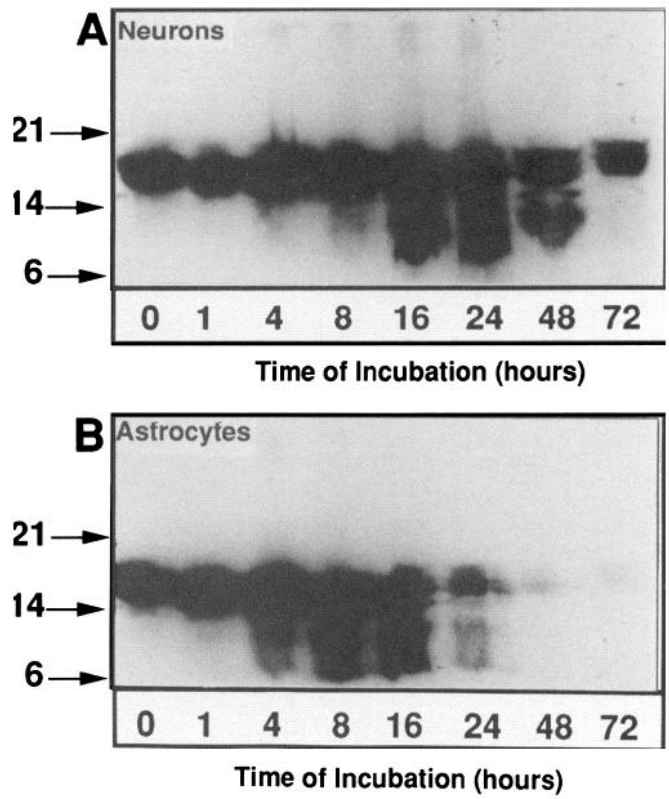

Figure 4. Time course of ^{125}I–basic FGF metabolism. Hippocampal neurons (*A*) and astrocytes (*B*) were incubated with ^{125}I–basic FGF at 4°C for 4 hr, washed as described in the text, and further incubated at 37°C for the number of hours indicated. The metabolism of basic FGF was assessed by autoradiography of the extracts after SDS-PAGE. Molecular weights (in kDa) are indicated to the *left*.

pectedly (Bouché et al., 1987; Baldin et al., 1990), label was not seen associated with the nucleolus but was detected overlying nuclear chromatin. For example, note the large collection of grains in the nucleus in the cell in Figure 2*A*. Label was never seen in association with mitochondria.

In hippocampal neurons (Fig. 2*C*), autoradiographic grains are seen in the midst of the nuclear chromatin and near the margin of the cell. In Figure 2*D*, label is seen in association with a neuritic process, in a cytoplasmic vesicle, and in the nuclear chromatin near the nuclear membrane. As with astrocytes, the most frequently labeled structures are cytoplasmic vesicles and lysosomes. Higher magnification (Fig. 2*E,F*) reveals the second most frequent site of autoradiographic grains, over the cytoplasm near vesicular structures. Finally, as with astrocytes, the third most common site is associated with the nuclear chromatin. Label was not detected over mitochondria or the nucleolus.

Biochemical analyses of ^{125}I–basic FGF binding. In other studies (Walicke et al., 1989), we have compared the high- and low-affinity receptors on neurons and astrocytes. Because the autoradiograms of labeled cells suggest that the basic FGF added to cells is long-lived, we attempted to perform a biochemical analysis of the ^{125}I–basic FGF. Extraction and processing of the cells immediately after the binding incubation revealed the presence of an 18-kDa doublet (Fig. 3, lanes 1, 4). After 16 hr at 37°C (Fig. 3, lanes 3, 6), both neurons and astrocytes contain several smaller peptides ranging from 4 to 16 kDa. These peptides are all detectable in extracts of the astrocytes 4 hr after binding (Fig. 3, lane 5). An analysis by SDS-PAGE and autoradiography of the neuron extract, however, shows that a majority of the label is in the 15.5-kDa protein at this time (Fig. 3, lane 2). The appearance of the smaller peptide coincides with the disappearance of the basic FGF doublet. The dimerized form of basic FGF that is generated by storage (P. A. Walicke and A. Baird, unpublished observations) is readily detected in the starting material (Fig. 3, lanes 1, 4) This dimer, which cannot be reduced under the conditions of reducing SDS-PAGE, has a molecular weight of approximately 36 kDa and is observed at time 0 (Fig. 3, lanes 1, 4). Both neurons and astrocytes utilize this basic FGF dimer, as demonstrated by its disappearance after 4 hr in astrocytes (Fig. 3, lane 5) and its metabolism after 16 hr in neurons (Fig. 3, lane 3). Accordingly, the basic FGF dimer is most likely active, a conclusion compatible with the fact that dimerization of standard basic FGF preparations does not decrease specific activity (Walicke and Baird, unpublished observations).

The processing of basic FGF over 3 d in culture is shown in Figure 4. In extracts of neurons (Fig. 4*A*), the metabolism of basic FGF is barely detectable at 4–8 hr, but is robust at 16 hr. Between 48 and 72 hr after binding, there are no longer any metabolic products detectable, though there remain significant amounts of unprocessed basic FGF. In astrocytes (Fig. 4*B*), the smaller peptides are first detected after the cells have been returned to 37°C for 4 hr. They are found in increasing amounts when the cells are further incubated from 8 to 16 hr. The

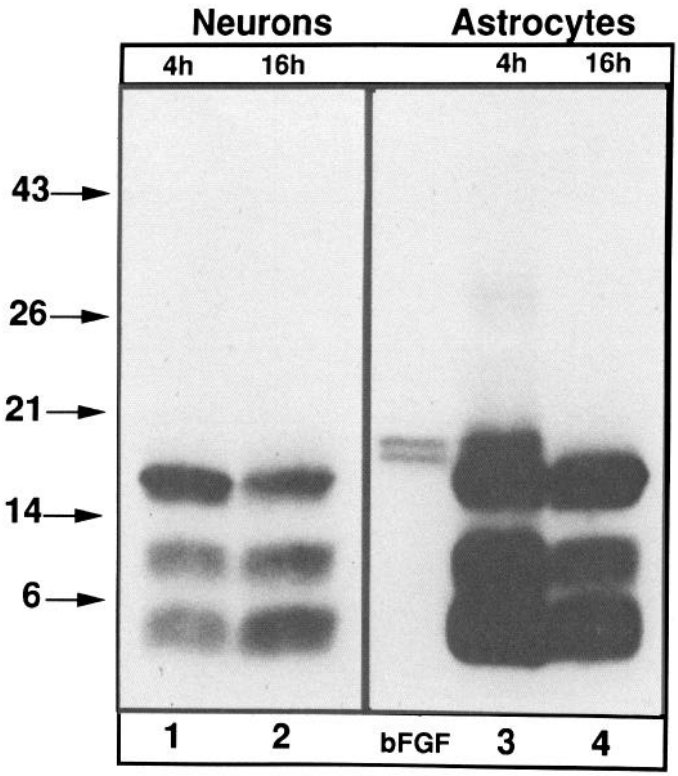

Figure 5. Heparin binding of the metabolized products of ^{125}I–basic FGF. SDS-PAGE and autoradiography was performed on the dialyzed 2 M NaCl eluate of heparin-Sepharose column chromatography of hippocampal neurons (*lanes 1, 2*) or astrocytes (*lanes 3, 4*) extracted 16 hr after binding ^{125}I–basic FGF. Molecular weights (in kDa) are indicated to the *left*. The unlabeled autoradiographic lane is intact ^{125}I–basic FGF.

radiolabeled peptides then disappear after 24 hr and are virtually undetectable by 48 hr.

Characterization of the metabolic products. Because the labeled peptides detected in the cell extracts could be generated from free ^{125}I-tyrosine rather than being fragments of ^{125}I–basic FGF, cells were incubated with radiolabeled basic FGF for 16 hr at 37°C after binding, and the extracts were then applied to a heparin-Sepharose column. The peptides eluting between 1.0 and 2.0 M NaCl were examined by SDS-PAGE and autoradiography. At least three heparin-binding peptides were present in this fraction (Fig. 5). The decreased background and the higher resolution made it possible to establish that the basic FGF doublet is metabolized to a slightly lower-molecular-weight species of 15.5 kDa and at least two other smaller heparin-binding fragments of 5–10 kDa.

These putative degradative fragments of basic FGF can be immunoprecipitated with a panel of antisera to confirm their derivation from basic FGF (Fig. 6). Astrocytes incubated with labeled basic FGF for 16 hr show three major metabolic products (Fig. 6, lane 2). A series of six antisera were tested for their ability to immunoprecipitate these fragments: anti–basic FGF[1–24], anti–basic FGF[30–50], anti–basic FGF[69–87], anti–basic FGF[106–120], anti–basic FGF[139–146], and an antiserum raised against bovine serum albumin. The last was used as a control because antisera were generated against peptides conjugated to BSA. All anti–basic FGF antibodies immunoprecipitate intact basic FGF (not shown). The 15.5-kDa protein is precipitated by four of the five antisera tested. The only antiserum that failed to immunoprecipitate this band was anti–basic FGF[139–146], suggesting that the first step in metabolism of basic FGF is the removal of the carboxyl terminal. The 9-kDa peptide was precipitated with anti–basic FGF[30–50] and anti–basic FGF[69–87], but not with anti–basic FGF[1–24] or anti–basic FGF[139–146]. Thus, this fragment contains a midportion sequence, somewhere between basic FGF[25–105]. Because the 4-kDa band could only be precipitated with anti–basic FGF[106–120], it is most likely a C-terminal fragment. Identical results

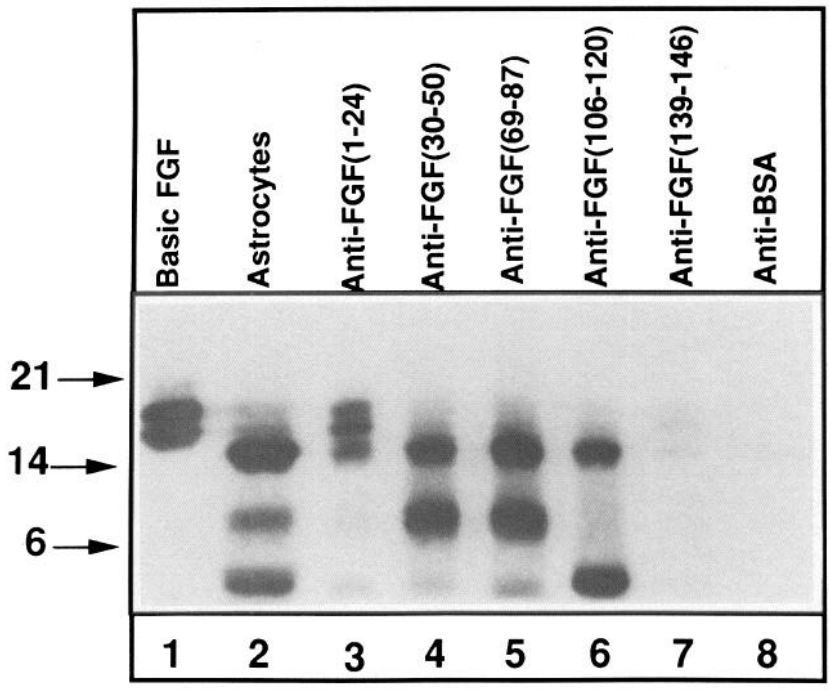

Figure 6. Immunoprecipitation of ^{125}I–basic FGF metabolized by astrocytes. The cells were extracted 16 hr after the binding assay, and the 2 M NaCl eluate from a heparin-Sepharose affinity column was immunoprecipitated with various antisera to basic FGF. *Lane 1,* intact ^{125}I–basic FGF; *lane 2,* astrocyte cell extract; *lane 3,* precipitation with anti–basic FGF[1–24]; *lane 4,* precipitation with anti–basic FGF[30–50]; *lane 5,* precipitation with anti–basic FGF[69–87]; *lane 6,* precipitation with anti–basic FGF[106–120]; *lane 7,* precipitation with anti–basic FGF[139–146]; *lane 8,* precipitation with antiserum to bovine serum albumin. Molecular weights (in kDa) are indicated to the *left*.

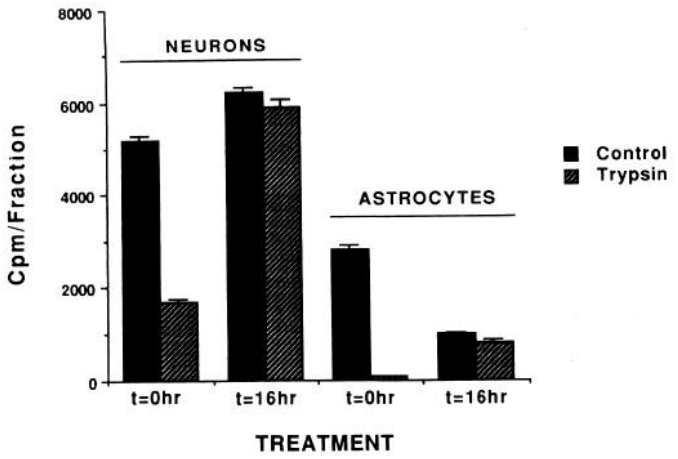

Figure 7. Effect of trypsin/NaCl on the recovery of ^{125}I–basic FGF from cells. Cells were incubated with radiolabeled basic FGF and processed immediately or after 16 hr at 37°C. *Solid bars* show whole culture extracts; *hatched bars* show extracts after cultures were treated with 0.25% trypsin for 5 min at 37°C and washed with 2 M NaCl prior to extraction. The extract was analyzed by step-gradient elution of heparin-Sepharose affinity columns, and the counts of the 1–2 M eluate are shown. Figures are means ± SEM for triplicate samples.

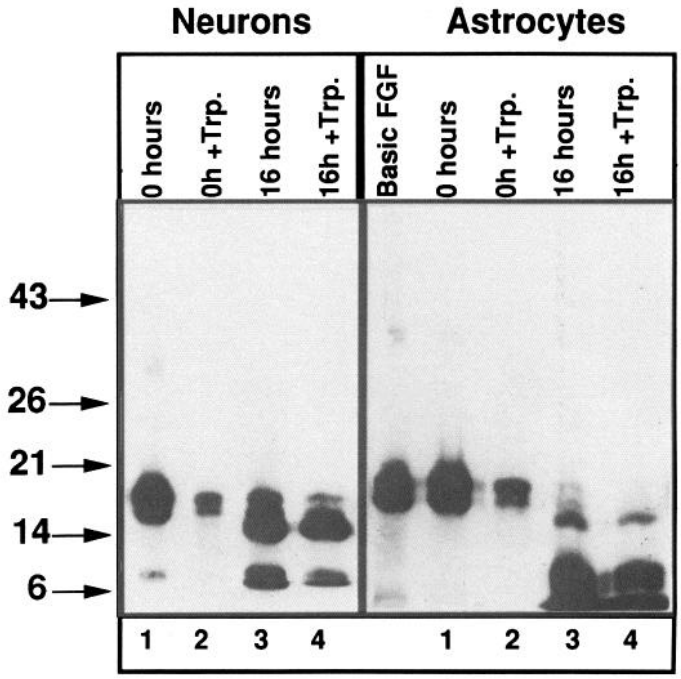

Figure 8. Effect of trypsin/NaCl on the metabolism of ^{125}I–basic FGF. Cultures were prepared as described in the text. ^{125}I–basic FGF incubated with media but with no cells is shown in the *middle. Lanes 1,* cells extracted immediately after the binding assay; *lanes 2,* cells treated with trypsin/2 M NaCl immediately after binding; *lanes 3,* cells extracted 16 hr after binding; *lanes 4,* cells treated with trypsin/2 M NaCl 16 hr after binding. Molecular weights (in kDa) are indicated to the *left.*

were obtained with peptides extracted from neurons (data not shown).

Localization of basic FGF metabolism. Most cells, including astrocytes and neurons, are known to secrete proteases into the culture medium. Therefore, the metabolism of basic FGF could potentially occur before, during, or after internalization. To investigate this possibility, the extraction procedures were modified to determine whether the labeled peptides were metabolized extracellularly or intracellularly. After the binding assays, cells were first treated with trypsin to remove extracellular ^{125}I–basic FGF and to degrade membrane receptors (Moscatelli, 1987, 1988; Moenner et al., 1989). Cells were then washed with 2.0 M NaCl to remove any basic FGF bound to extracellular GAGs. Finally, the cells were extracted. As expected (Walicke et al., 1989), the combined treatment with trypsin and 2 M NaCl removed 70–90% of the total basic FGF that was bound to the neurons and astrocytes if performed immediately after binding (Fig. 7). When the procedure was repeated on neurons or astrocytes that had been incubated a further 16 hr at 37°C, the amount of radioactivity recovered was the same with or without the trypsin and 2 M NaCl treatment. Thus, much more of the label is protected from extracellular proteases and salt 16 hr after binding, supporting the interpretation that the peptide fragments are intracellular. The treatments with trypsin and NaCl 16 hr after the binding assay had no effect on the general pattern of iodinated basic FGF metabolism, though signal intensity is decreased (Fig. 8).

Metabolism of basic FGF is mediated by its receptor. All studies performed to date with mesenchymal or neuroectodermal cells suggest that there are two components of basic FGF binding, a low-affinity component related to a cell-surface GAG and a glycoprotein high-affinity receptor. Accordingly, GAGs generally bind most of the ^{125}I–basic FGF added to cells. The time-course studies show that about 75% of the counts that bind to cells at 4°C shift to an intracellular compartment when the cells are returned to 37°C for 16 hr (not shown). Because only 30–50% of this label is associated with high-affinity receptors during the binding assay (Walicke et al., 1989), the results suggest that, over the course of the following 16-hr incubation, the basic FGF associated with low-affinity receptors (i.e., GAG associated) is eventually internalized. To examine this possibility, the binding of ^{125}I–basic FGF to GAGs was inhibited with heparin (Walicke et al., 1989), and the binding of ^{125}I–basic FGF to high-affinity receptors was inhibited with WGA (Feige and Baird, 1988).

Cells were exposed to ^{125}I–basic FGF in the presence of 100 μg/ml of either heparin or WGA during both the binding assay and the subsequent 16-hr incubation at 37°C, and SDS-PAGE autoradiography was performed on cell extracts to determine whether the degradative peptides were formed. As shown in Figure 9, the labeled fragments were detected in extracts of the untreated (Fig. 9, lanes 1, 4) and heparin-treated (lanes 2, 5) astrocytes and neurons. Processing of the cells that were incubated with WGA (lanes 3, 6) reveals the presence of very little peptide (intact basic FGF is readily detectable). Thus, the peptides are generated from the ^{125}I–basic FGF internalized with their receptors. In contrast, though heparin decreased the intensity of the metabolized products, they were readily detectable.

Discussion

The studies reported here suggest that neuroectodermal cells internalize and metabolize basic FGF in a manner similar to mesenchymal cells (Moenner et al., 1987, 1989; Moscatelli, 1988). After internalization, basic FGF is processed into several specific peptides that remain associated with the cells for long periods after binding. This processing is distinctly different than observed for most polypeptide growth factors that appear to be metabolized within minutes of internalization. The detection of

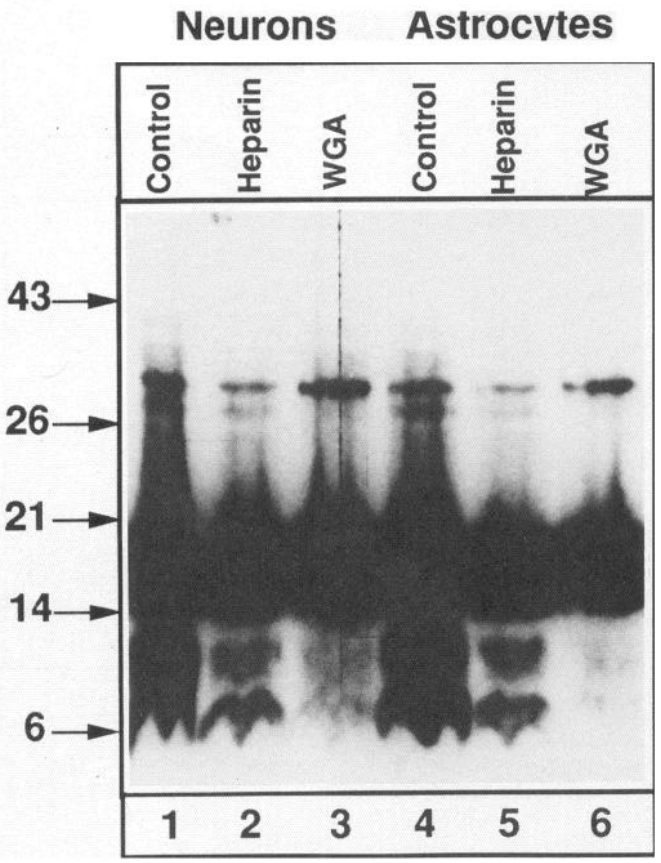

Figure 9. Effect of exogenous heparin and WGA on the metabolism of ^{125}I–basic FGF. Neurons (*lanes 1–3*) or astrocytes (*lanes 4–6*) were incubated for 16 hr at 37°C after the binding assay and then extracted as described in the text. *Lanes 1* and *4*, control; *lanes 2* and *5*, + 100 μg/ml heparin; *lanes 3* and *6*, + 100 μg/ml WGA. Heparin or WGA were present during the binding and subsequent incubation period. Molecular weights (in kDa) are indicated to the *left*. The 30-kDa doublet is the dimerization of basic FGF that occurs during storage (Walicke and Baird, unpublished observations).

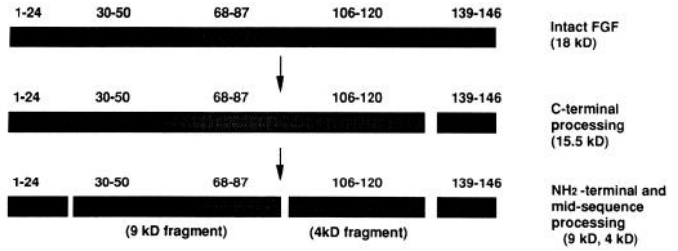

Figure 10. Proposed metabolism of basic FGF by neurons and astrocytes. Based on the molecular weights of the immunoprecipitated metabolic products of basic FGF, a scheme can be outlined whereby the processing of basic FGF occurs initially at the C-terminus and is followed by amino terminal cleavage to yield a radiolabeled C-terminal fragment.

basic FGF in the nucleus supports the elegant studies of Bouché and coworkers (Bouché et al., 1987; Baldin et al., 1990). However, we consistently failed to see basic FGF in the nucleolus. Rather, our results support the findings of several investigators (Logan, 1990; Renko et al., 1990) who have suggested that basic FGF can be translocated to the nucleus, where it has further activities. The findings reported here support this notion and suggest that there exists a specific interaction between basic FGF (or a metabolic fragment) and chromatin. To our knowledge, this is the first demonstration of the internalization of basic FGF into the nucleus of a CNS cell. Perhaps even more importantly, the results establish the fact that basic FGF acts directly on neurons.

The metabolic fragments of basic FGF produced by CNS cells were not isolated and sequenced. Even so, several inferences about their identity can be made. These are summarized in Figure 10. The 15.5-kDa peptide lacks the normal carboxyl terminus, a transformation that may alter heparin binding activity (Seno et al., 1990). The 9-kDa peptide contains sequences found between amino acids 30 and 87, but its apparent molecular weight suggests that it may include a somewhat larger region. Failure to immunoprecipitate this peptide with the antiserum to basic FGF(1–24) establishes that the amino terminus has been deleted. Accordingly, the predicted peptide has the protein kinase C (PKC) consensus sequence present in basic FGF (Feige et al., 1989; Feige and Baird, 1991) at residue Ser^{64}. The apparent molecular weight of the 4-kDa fragment that contains the 106–120 epitope suggests that it contains further portions of the basic FGF sequence, possibly as much as basic FGF(87–139). This peptide contains the consensus sequence for protein kinase A (PKA)-dependent phosphorylation (Feige et al., 1989; Feige and Baird, 1991) and a cryptic site for PKC (Feige et al., 1991) that is phosphorylated on peptide fragments but not on intact basic FGF. Because the degradation to these fragments may generate cryptic heparin binding and phosphorylation sites that could confer activity, it will be imperative to identify the fragments.

Several investigators have proposed that basic FGF bound by extracellular matrix serves as a stable reservoir in tissues (Baird and Ling, 1987; Vlodavsky et al., 1987; Folkman et al., 1988; Baird and Walicke, 1989). It was thus interesting to note that one interpretation of the results obtained with exogenous heparin and WGA is that basic FGF is delivered to the receptor by cell-associated GAGs. When WGA inhibits high-affinity receptor binding, it selectively inhibits the metabolism of basic FGF. As expected, the amount of ^{125}I–basic FGF internalized is accordingly decreased. When heparin is used to inhibit the binding to low-affinity receptor, the metabolism of basic FGF is unaffected, but there is a significant decrease in the amounts of label internalized.

The intracellular metabolism of basic FGF generates two major fragments of basic FGF that contain the middle portion of the molecule and thus the receptor binding sites (Baird et al., 1988). This specific pattern of metabolism coupled with the long-lived nature of the peptide fragments generated raises the interesting possibility that the peptides can serve as intracellular and even intranuclear (Baldin et al., 1990; Renko et al., 1990) signals. It is thus of more than passing interest that the fragmentation pattern cleaves basic FGF into one peptide that is a substrate for the phospholipid-dependent kinase PKC and a second peptide that is a substrate for the cAMP-dependent kinase PKA (Feige and Baird, 1989, 1991; Feige et al., 1989, 1991). With this knowledge, it is possible that the phosphorylation of basic FGF is involved in the processes that mediate basic FGF activity, a possibility that can easily be addressed by site-directed mutagenesis.

It is also important to note that, though equal cell numbers were used in these experiments, astrocytes consistently metab-

olized basic FGF faster than neurons. Although the significance of this finding is not clear, it may reflect the differences between the FGF receptor of each cell type (Walicke et al., 1989). On this basis, it is interesting to speculate that the measurement of these basic FGF fragments in the CNS could be used to determine whether the neurotrophic factor is being metabolized *in vivo*. The recent observations of Carmen et al. (1990) support such a hypothesis. These investigators examined the fate of basic FGF injected into the CNS and found that, while at the time of injection basic FGF binds to the parenchyma, it diffuses along adjacent fiber tracts and is present for at least 7 d as an 18-kDa protein. Four days after the injection, however, the presence of the same metabolic fragments as those described here are detected (A. Gonzales and A. Bird, unpublished observations). The availability of antisera to measure these fragments specifically could thus permit investigators to study the kinetics of basic FGF metabolism during CNS injury, ischemia, and perhaps even in the neurodegenerative disease in which basic FGF has been implicated (Stopa et al., 1990). This approach would thus enable investigators to distinguish between ubiquitous presence of the mitogen when it is being sequestered in tissues and its presence when it is being actively metabolized.

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