Basic Fibroblast Growth Factor Enhances Nerve Growth Factor Receptor Gene Promoter Activity in Human Neuroblastoma Cell Line CHP100

MUTSUO TAJI,† KAZUKO TAJI,† KAREN L. DEYERLE,‡ AND MARK BOTHWELL*

Department of Physiology and Biophysics, School of Medicine, University of Washington, Seattle, Washington 98195

Received 29 May 1991/Accepted 11 February 1992

The human neuroblastoma cell line CHP100 provides a useful model system in which to study the molecular mechanisms of transcriptional regulation of the low-affinity nerve growth factor receptor (NGFR) gene during neuronal development. Basic fibroblast growth factor (bFGF) induced morphological changes in CHP100 cells, including flattening of cell bodies and neurite outgrowth. bFGF also increased p75NGFR immunoreactivity, as assessed by immunocytochemistry, and increased p75NGFR mRNA levels, as assessed by Northern (RNA) blot analysis. A chimeric gene consisting of 6.7 kb of the 5'-flanking region of the human NGFR gene linked to the chloramphenicol acetyltransferase gene was constructed. In stable transformants of CHP100 cells, 10 ng of bFGF per ml induced an eightfold increase in chloramphenicol acetyltransferase activity. These results indicate that upstream elements of the NGFR gene mediate transcriptional regulation by bFGF.

Growth factors and their cognate receptors show highly programmed patterns of expression and play crucial regulatory roles in tissue growth and differentiation during vertebrate embryogenesis. Recent evidence suggests that development of the peripheral nervous system is under the control of a variety of growth factors. Basic fibroblast growth factor (bFGF) and acidic FGF have been shown to initiate the differentiation of neonatal chromaffin cells into immature sympathetic neurons, which in turn require nerve growth factor (NGF) for survival and maturation (15, 57).

In a v-myc-immortalized sympathoadrenal precursor cell line, bFGF was found to elevate levels of mRNA encoding the 75-kDa low-affinity NGF receptor (p75NGFR) (5). On the basis of these studies, Anderson and coworkers have proposed that bFGF (or a similar factor) and NGF act sequentially during differentiation of sympathetic neurons. Recent studies in our laboratory have demonstrated that the timing of expression of receptors for bFGF and NGF in developing chicken embryo sympathetic ganglia is consistent with the proposed sequential action of these factors. Embryonic sympathetic ganglia cells initially express relatively high levels of FGF receptor mRNA but only low levels of p75NGFR mRNA. Subsequently, levels of p75NGFR mRNA increase while expression of FGF receptor mRNA ceases (30).

NGF is a member of a family of structurally related neurotrophic factors (neurotrophins), including brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), neurotrophin-4 (NT-4), and neurotrophin-5 (NT-5) (for recent reviews, see references 3, 27, and 31). Additional members of the trk gene family, trkB and trkC, encode receptors for BDNF, NT-3, NT-4, and NT-5 (3, 31, 36, 37, 55, 56). A functional role for p75NGFR in facilitating cellular actions of BDNF, NT-3, NT-4, and NT-5 mediated by trkB and trkC receptors has been suggested (7) but not yet demonstrated.

Although many experiments have been carried out using rat pheochromocytoma (PC12) cells as a model for sympathetic neuronal differentiation, these cells have substantial levels of p75NGFR expression in the absence of bFGF and attain equivalent degrees of neuronal differentiation in response to NGF and bFGF (47). Thus, they are not an suitable model of sympathetic neuronal stem cells with respect to some responses to bFGF and NGF. For this reason, we sought to identify an alternative in vitro system utilizing neuroblastoma cells which is more suitable for study of some elements of the mechanism of sympathetic neuronal differentiation. Owing to their neural crest origin, various human neuroblastoma cell lines have been used as models for peripheral neuron differentiation (44). Several of these cell lines have been shown to be capable of undergoing neuronal differentiation following induction by factors such as NGF, retinoic acid, phorbol ester, cyclic AMP analogs, and protein kinase inhibitors (28, 45, 53, 54). One of these cell lines, CHP100 (50), has been assigned to the subgroup of primitive neuroectodermal tumors which has cholinergic rather than adrenergic properties (46). In this respect, these cells may resemble parasympathetic neurons or the cholinergic subpopulation of sympathetic neurons. However, our own unpublished results demonstrate that these cells also express the adrenergic marker, tyrosine hydroxylase. The coexpression of cholinergic and adrenergic traits is consistent with the behavior of immature sympathetic neurons (43). Northern (RNA) blot and Western blot (immunoblot) analyses have shown that CHP100 cells express the endogenous NGF receptor (NGFR) gene (2). However, as demonstrated in the present report, CHP100 cells express only relatively low levels of p75NGFR, and expression of p75NGFR is substantially enhanced by bFGF. Thus, the CHP100 cell line provides a useful system for study of bFGF-initiated
neuronal differentiation and stimulation of NGFR gene expression.

In the present study, using CHP100 cells as a model system for sympathetic neuron development, we have employed immunocytochemistry. Northern blot analysis, and transfection of NGFR gene promoter-reporter gene constructs to study the role of NGF and FGF in neuronal differentiation and regulation of expression of p75NGFR. We show that CHP100 cells respond to bFGF treatment with morphological differentiation and increased expression of p75NGFR protein. The latter response appears to result, in substantial part, from an increased steady-state level of p75NGFR mRNA. A previous study has demonstrated that 5'-flanking sequences of the human NGFR gene possess a strong constitutive promoter activity (52). The present study demonstrates that this promoter activity is subject to regulation by bFGF in CHP100 cells.

MATERIALS AND METHODS

Cell culture. CHP100 neuroblastoma cells (generously provided by H. L. Schlesinger (50)) were cultured in Dulbecco’s modified Eagle’s medium (GIBCO) supplemented with 10% fetal calf serum (Hazelton) and 4 mM L-glutamine (GIBCO). Cells were trypsinized and seeded at 1 x 10^2 to 2 x 10^6 cells per cm² onto noncoated dishes. Recombinant human bFGF (gift of Chiron Corporation) was added at the time of plating, and fresh bFGF was added every 2 to 3 days. bNGF was purified from mouse submaxillary glands by the method of Burton et al. (10).

Immunocytochemistry. CHP100 cells were plated at 1 x 10^5 cells per cm² onto gelatin-coated glass slides and cultured in a humidified incubator. To study the effect of bFGF, 10 ng of bFGF per ml was directly added to the culture. Cells were fixed with cold methanol at -20°C for 10 min. Immunocytochemical staining for p75NGFR was performed essentially as described previously (49, 59). Briefly, cells were washed three times with phosphate-buffered saline (PBS) and incubated with 3% horse serum and 2% fetal calf serum in PBS at 1 h at room temperature. A monoclonal antibody against human p75NGFR, NGFRs (39), was used as the primary antibody to detect human p75NGFR, and a monoclonal antibody against rat p75NGFR, mc192, (11) was used as control. These primary antibodies (5 µg/ml) were applied for 4 h at 4°C onto gelatin-coated plates and then overnight at 4°C. All subsequent procedures were carried out at room temperature. Cells were washed with PBS and incubated with biotinylated horse anti-mouse immunoglobulin G (7.5 µg/ml; Vector) for 1 h. Cells were washed again and treated with streptavidin-peroxidase (Zymed Laboratories) and then with 3,3'-diaminobenzidine tetrahydrochloride (0.04%; Sigma), nickel sulfate (2.5%; Sigma) and H_2O_2 (0.3%) in sodium acetate buffer (pH 6.0) according to the instructions of Zymed Laboratories.

ELISA. Enzyme-linked immunosorbent assay (ELISA) of p75NGFR was performed essentially as described by Doherty et al. (21). Briefly, 6,000 CHP100 cells were plated in gelatin-coated wells of 96-well plastic tissue culture plates (Corning) and incubated with various concentrations of bFGF for 2 days. After incubation, cells were fixed with 4% paraformaldehyde, and were reacted with 5 µg of ME20.4 antibody (Boehringer-Mannheim) per ml for 1 h at 20°C. Wells were washed and then incubated with 1 µg of horse-radish peroxidase-conjugated rabbit anti-mouse immunoglobulin G (Sigma) per ml for an additional 1 h. After additional washing, wells were incubated with 50 µl of 0.2% o-phenylenediamine and 0.02% hydrogen peroxide in citrate phosphate buffer. The optical density was spectrophotometrically determined at 490 nm.

Northern blot analysis. Cytoplasmic RNA was extracted from CHP100 cells or NB6704 cells by the method of Sambrook et al. (48). Briefly, cells were treated with 0.5% Nonidet P-40 and centrifuged to obtain nucleus-free supernatants, from which cytoplasmic RNA was purified by phenol-chloroform extraction. The RNA was fractionated on 1% agarose gels containing 6% formaldehyde and then electrophoreted onto GeneScreen membranes (NEN). The membrane was soaked in methylene blue solution to stain rRNA in order to verify that equivalent amounts of RNA were transferred to the membrane in each lane. Prehybridization was performed on the membrane at 43°C for 16 h in a solution containing 50% formamide, 1x Denhardt’s solution, 1.0 M NaCl, 1.0% sodium dodecyl sulfate (SDS), 10% dextran sulfate, 50 mM Tris-HCl (pH 7.5), and 100 µg of denatured salmon sperm DNA per ml. The membrane was then hybridized at 43°C for 2 h in the same solution containing 2 x 10^6 cpml/m of 35S-labelled p75NGFR CDNA probe (Spnh1-HindIII 0.9-kb fragment; 10^6 cpml/m) or 2 x 10^6 cpml/m of 35S-labelled CAT DNA probe (HindIII 1.6-kb fragment; 10^6 cpml/m). The membrane was washed in 2 x SSC (1x SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 65°C for 15 min twice, washed in 0.1x SSC at room temperature for 15 min and then exposed to Kodak XAR film at -80°C with an intensifying screen.

Construction of NGFR promoter-chloramphenicol acetyltransferase (CAT) DNA. λ7A2 phage contains a portion of the genomic NGFR DNA sequence encompassing approximately 5.5 kb 5' to the transcriptional start site (25) and was designated pBlol. The Rous sarcoma virus (RSV) promoter, which contains the NGFR sequences as an EcoRI insert, was used as the starting material for the NGFR promoter constructs. The 4.3-kb BamHI fragment containing the NGFR promoter region (Fig. 1A) was isolated from λ7A2 and inserted into the unique BamHI site of pUC19. This plasmid was designated pB101. The AvaiII site at nucleotide -43, located between the mRNA cap site (−121 and −122) and the initiator methionine codon (+1) (45), was then converted to a SalI site via limited digestion of pB101 with AvaiII, treatment with mung bean nuclease, and subsequent ligation of SalI 10-mer linkers (CGGTCGACCG: Stratagene, catalog no. 90174) (Fig. 1B). Following ligation of SalI linkers, pB101 was digested with SalI, and the 6.5-kb fragment was isolated and recircularized (Fig. 1C). This new plasmid, designated pRP2, contains the 3.8-kb NGFR promoter as a BamHI-SalI fragment, while lacking the first exon and the 5' portion of the first intron of the NGFR gene. It should be noted that pRP2 contains a HindIII site (from the pUC19 polynucleotide) immediately downstream of the SalI site on the 3' boundary of the NGFR promoter (Fig. 1C). This plasmid also contains an NdeI site in the pUC19 sequences 5' to the NGFR promoter insert (Fig. 1C).

pRSV-CAT (25) was modified to provide the vector for the NGFR promoter. In this plasmid, the Rous sarcoma virus (RSV) promoter is bordered on the 3' side by a HindIII site, while upstream of the RSV promoter is an NdeI site contained within the pBR322 sequences incorporated into the vector (25) (Fig. 1D). In addition, a BamHI site is located in the vector sequences downstream of the CAT gene (Fig. 1D). The RSV promoter region was excised from pRSV-CAT as an NdeI-HindIII fragment and replaced with the pRP2 NdeI-HindIII fragment containing the NGFR promoter (Fig. 1E). This plasmid was designated pRP2C. The
NGF RECEPTOR INDUCTION BY FGF

RESULTS

Morphological changes of CHP100 cells induced by bFGF.

Diversity in cell morphologies is a hallmark of cultured neuroblastoma cells. Consistent with this, the cells of the neuroectodermal tumor line CHP100 display two distinct morphologies. As defined by others (4, 14), the majority of cells (approximately 75%) adopt a neuroblastic cell (N-type) phenotype, being small and teardrop-shaped with short processes and loosely adherent cell bodies (Fig. 2A). The remaining cells display an intermediate (I-type) morphology, having small and slightly flattened cell bodies (Fig. 2A). These two cell types are interconvertible during culture, and this interchange is sensitive to the culture conditions.

bFGF was found to induce profound morphological changes in CHP100 cells. After 24 h of incubation in medium containing 10 ng of bFGF per ml, some rapidly dividing round cells appeared. These cells were loosely attached to the monolayer of cells, eventually detached from the monolayer, and died within 48 h in the continued presence of bFGF. The remaining cells survived and ceased to divide but remained attached to the substrate. After 48 h of incubation with bFGF, these postmitotic cells possessed large flattened cell bodies, with some exhibiting enhanced elaboration of thick processes (Fig. 2C). More marked morphological changes were observed after 96 h of incubation with bFGF (Fig. 2D). At this time, all cells were found to be more adherent to the substrate, with some of the cells continuing to extend thick processes. The proportion of these latter cells was greater when cells were plated at lower cell density. It was noted that regular replacement of the medium with medium containing additional bFGF was necessary to maintain these morphological changes; otherwise, weakly responding cells started dividing again and covered most of the substrate. NGF did not induce any morphological changes in CHP100 cells (Fig. 2B) nor did NGF affect the morphology of bFGF-treated cells (data not shown).

Similar experiments were conducted with an independently derived CHP100 subclone which had a reversed ratio of the two cell types: the proportions of N- and I-type cells were approximately 10 and 90%, respectively. Despite this difference, these cells showed nearly identical responses to bFGF. The number of cells surviving 24 h of culture with bFGF was 39% ± 9% of that of untreated cells, which is in
agreement with the value obtained for the original CHP100 cells (39% ± 4%). Thus, it appears that both of the cell types (N and I types) are capable of responding to bFGF with similar morphological changes.

Cell morphologies of CHP100 cells and morphological changes induced by bFGF were influenced by the culture substrate. Most of the experiments described below employed noncoated tissue culture plastic substrata. However, for immunocytochemical analysis, a more adhesive substrate was required. We employed gelatin for this purpose, because cell morphologies on this substratum resembled morphologies on noncoated plastic more closely than did cells grown on other substrata. However, morphological responses to bFGF appeared more slowly on gelatin than on noncoated plastic.

**bFGF enhances the endogenous expression of p75NGFR in CHP100 cells.** Immunocytochemistry was used to examine the expression of endogenous p75NGFR in untreated and bFGF-treated CHP100 cells. Using the anti-human p75NGFR monoclonal antibody NGFR5 (39), very weak immunostaining was observed in untreated CHP100 cells (Fig. 3B). While weak, this staining was significant when compared with negative control cells (Fig. 3A) immunostained with monoclonal antibody mc192 (11). The latter antibody is directed against rat p75NGFR and cross-reacts poorly with human p75NGFR. Thus, we conclude that untreated CHP100 cells express their endogenous p75NGFR at low, but detectable, levels. In contrast, CHP100 cells treated with 10 ng of bFGF per ml for 48 h were stained significantly more strongly with NGFR5 antibody than were untreated control cells (Fig. 3C and D). Because of the generally slower FGF induction of morphological responses of cells cultured on gelatin, the morphological response of these cells was not pronounced by 48 h. Nevertheless, significant elevation of p75NGFR levels were observed, even on round cells which had not yet extended processes. The cell bodies of both round cells and differentiating cells and processes of differentiating cells were uniformly reactive with NGFR5 antibody. Similar results were obtained using another monoclonal antibody, NGFR2 (39) directed against a different p75NGFR epitope, confirming that the bFGF-induced p75NGFR-like immunoreactivity reflects authentic p75NGFR.

A more quantitative determination of the extent of induction of NGFR protein was accomplished using an enzyme-linked immunoassay. CHP100 cells plated on gelatin in 96-well plates were exposed to various concentrations of bFGF for 2 days, and then the quantity of p75NGFR was determined immunochemically, as described in Materials and Methods. This assay revealed a threefold increase in the quantity of receptor protein per dish (Fig. 4). The presence of bFGF caused a twofold reduction in the number of cells per well. Thus, bFGF causes an approximately sixfold increase in the quantity of receptor protein per cell.

**p75NGFR mRNA levels are enhanced by bFGF in CHP100 cells.** The increase in p75NGFR detected by immunochemical methods could result from several different processes, including enhanced gene transcription, increased mRNA or protein stabilization, and enhanced translation. In order to
FIG. 3. Immunocytochemical staining of CHP100 cells. CHP100 cells were grown without bFGF (A and B) or with 10 ng of bFGF per ml (C and D) for 2 days and then stained with antibody mc192 immunoglobulin G against rat p75NGFR (A and C) or with antibody NGFR5 against human p75NGFR (B and D). Nontreated CHP100 cells were slightly stained with antibody C34C. bFGF enhanced staining with antibody against p75NGFR in CHP100 cells. Bar, 50 μm.

FIG. 4. Assessment of bFGF-enhanced p75NGFR expression by ELISA. CHP100 cells were cultured for 2 days in the presence of the indicated concentrations of bFGF, and then the amount of p75NGFR was determined by ELISA, as described in Materials and Methods. O.D., optical density.

begin to distinguish among these possibilities, the effect of bFGF treatment on the steady-state level of p75NGFR mRNA was examined by Northern blot analysis. Cytoplasmic RNA was extracted from CHP100 cells following 48 or 96 h of incubation with or without bFGF, and p75NGFR mRNA was detected through hybridization with a 32P-labelled 0.9-kb p75NGFR cDNA fragment. A single faint 3.8-kb transcript was detected in untreated CHP100 (Fig. 5). The size of this transcript is consistent with previous reports, which also detected a single 3.8-kb transcript in several different human neuroblastoma cells (2) and in a variety of primate tissues (49). This 3.8-kb p75NGFR message was found to be increased in CHP100 cells following bFGF treatment (Fig. 5). Densitometric analysis of autoradiographic bands revealed a 4.1-fold increase after 2 days and a 4.5-fold increase after 4 days with 10 ng of bFGF per ml. NGF did not have any significant effect. These data indicate that the elevated level of p75NGFR protein in bFGF-treated CHP100 cells is due, at least in part, to increased levels of p75NGFR mRNA. As for previous experiments, bFGF inhibited cell proliferation and reduced the final number of CHP100 cells, compared with that of controls, so the increase of p75NGFR mRNA per cell is substantially greater than fourfold.

bFGF enhances CAT activities in stably transformed CHP100 cells. To determine whether bFGF-induced elevation of p75NGFR mRNA levels results from activation of the NGFR gene promoter, we placed the reporter CAT gene under the transcriptional control of a 6.7-kb NGFR 5' flanking region known to contain elements of the NGFR
promoter (52) (Fig. 1). This expression vector was designated pRBLCAT. Preliminary experiments showed that a low level of CAT activity was obtained in CHP100 cells transiently transformed with pRBLCAT (data not shown). This DNA, together with ptkneo, a plasmid conferring resistance to the antibiotic neomycin, was then introduced by transfection into CHP100 cells. Stably transformed clonal lines were selected by culture in G418, and several clonal lines were characterized. One cell line, NB6704, which showed the highest basal CAT activity among the stable transformants, was used to study the NGFR promoter activity in detail. The limited basal expression was subsequently used to normalize the CAT activities of bFGF-treated transformed cells.

Figure 6 shows the relative CAT activities of extracts from NB6704 cells treated with different concentrations of bFGF for 24 h. bFGF is seen to increase CAT activity in a dose-dependent manner. Half-maximal CAT enhancement occurred at a dose of 3 ng of bFGF per ml, while 10 ng of bFGF per ml induced a maximum (approximately eightfold) of CAT activity. Experiments with several other cell lines stably transformed with pRBLCAT gave similar results, as did a pool of approximately 50 stably transformed colonies (data not shown).

A time course of CAT induction is shown in Fig. 7. NB6704 cells incubated with 10 ng of bFGF per ml showed increased CAT activity approximately 6 h posttreatment, with maximal activity being achieved following 24 h of treatment. A half-maximal increase in CAT activities was obtained after 12 h of incubation with bFGF.

To rule out the possibility that bFGF might somehow directly stimulate activity of the CAT enzyme, the level of CAT mRNA was examined through Northern blot analysis of NB6704 cell cytoplasmic RNA using a CAT DNA probe. Treatment of cells with 5 to 10 ng of bFGF per ml for 2 to 4 days yielded elevated levels of CAT mRNA to approximately the same extent as previously observed for NGFR mRNA (Fig. 8). The largest and most abundant transcript corresponds to the expected 1.6-kb CAT mRNA; two smaller transcripts, of unknown origin, were consistently observed, in constant proportion to the amount of 1.6-kb mRNA. These may possibly arise by RNA splicing or from downstream sites of transcription initiation. By densitometric analysis of the 1.6-kb message, increases of approximately two- and threefold were found to be induced by treatment with 5 and 10 ng of bFGF per ml for 48 h, respectively, while three- and fivefold increases were observed after treatment with 5 and 10 ng of bFGF per ml for 96 h, respectively.

The effect of bFGF on CAT activity was compared with that of NGF. Figure 9 shows the relative CAT activities of NB6704 cells treated for 24 h with 10 ng of bFGF per ml or...
FIG. 8. Northern blot analyses of CAT transcripts in NB6704 cells. Cytoplasmic RNA was prepared from NB6704 cells not treated (lane 1) or treated with 5 (lanes 2 and 4) or 10 (lanes 3 and 5) ng of bFGF per ml after 2 (lanes 1, 2, and 3) or 4 (lanes 4 and 5) days of incubation. Twenty milligrams of cytoplasmic RNA was loaded in each lane, separated on 1% agarose-6% formaldehyde gel, and hybridized to 32P-labelled CAT cDNA probe, as described in Materials and Methods. Arrows mark positions of 18S and 28S rRNA. A major band due to CAT mRNA was detected at 1.6-kb position (lane 1), and the intensity of the band increased with bFGF treatments (lanes 2, 3, 4, and 5).

100 ng of NGF per ml or both. bFGF was found to increase CAT activity eightfold above that of the control, whereas no significant increase in CAT activity was observed with NGF treatment. NGF did not significantly affect the enhancement of CAT activity induced by bFGF. The slight apparent increase with NGF in the data presented was not reproduced in other experiments.

DISCUSSION

bFGF induces neuronal differentiation of CHP100 cells and regulates p75NGFR expression. One of the fundamental strategies in the study of molecular mechanisms of ontogenetic processes is to identify factors which regulate cell proliferation and differentiation in early stages of development. One intriguing candidate for this role is bFGF. bFGF mRNA has been reported to be stored in unfertilized amphibian eggs (33), high levels of expression of bFGF have been found in early stages of mammalian and avian development (24, 32), and expression of FGF receptors is widely distributed in early stages of development (30, 42). A family of factors with substantial structural similarity to bFGF and acidic FGF has been described, including Int-2 (19), the kst gene product (8, 58), FGF-5 (63), FGF-6 (40), and keratinocyte growth factor (23). Several members of the FGF family, including acidic FGF and the kst gene product may act on cells employing the same receptor used by bFGF (20).

Among the potential roles of bFGF in embryonic development, bFGF or bFGF-related factors have been proposed to function in primary mesodermal induction (17, 33). Injection of antibodies to bFGF interferes with normal embryonic development, providing direct evidence for the involvement of bFGF in developmental regulation (38).

bFGF may play an important role as a mitogen and neurotrophic factor for the progenitor cells of both the central and peripheral nervous system (1, 30, 41, 51, 60, 61). In chick sympathetic neurons in vivo, high levels of FGFR mRNA have been observed in early developmental stages, suggesting a potential role for FGF in the initiation of differentiation (30). In vitro experiments have supported this suggestion. In rat sympathoadrenal progenitor cells, which have the ability to differentiate into adrenal chromaffin cells or sympathetic neurons, bFGF induces differentiation into sympathetic neurons, with a concomitant development of a requirement for NGF for survival (57). A v-myc-immortalized cell line of rat sympathoadrenal progenitor was used in further analyses of sympathetic neuronal differentiation, in which bFGF was found to induce growth of neuritic processes and increase the level of p75NGFR mRNA (5). These observations suggest that FGF is initially required for triggering the differentiation process and inducing p75NGFR expression. The development of neural crest-derived migratory precursor cells of sympathetic ganglia involves a deterministic interaction with the ventral neural tube and somitic mesenchyme (6, 43). It seems reasonable to suggest that this interaction may be mediated in part by bFGF or a similar factor.

Although NGF is also known to be an important factor for the development and maintenance of sympathetic neurons, NGF is not essential in early developmental stages but is required in later stages (6, 16, 43). In vitro studies have demonstrated that cultured chick sympathetic neurons taken from an early stage of development showed little response to NGF (22), and the appearance of adrenergic cells in culture was found to be independent of NGF (13). Indeed, sympathetic neurons may not be exposed to NGF until after target innervation is complete (35). All these observations are consistent with the view that NGF is involved only in the final stages of neuronal maturation, subsequent to bFGF-induced enhancement of NGFR expression. The finding that FGF receptors and NGFRs are sequentially expressed in the developing avian central nervous system as they are in the sympathetic nervous system (30) suggests that sympathetic neurons may also serve as a useful model for interactions between FGF and neurotrophic systems in the central nervous system.

In the present study, untreated CHP100 cells were found to express low levels of both p75NGFR protein (Fig. 3B and 4) and p75NGFR mRNA (Fig. 5), in agreement with previous observations (2). We have shown that bFGF induces marked morphological changes in CHP100 cells (Fig. 2). Two quite different patterns of response to bFGF are induced in separate subpopulations of cells. One subset of cells responds with enhanced proliferation rate and detachment from the substratum. The remaining bFGF-responding cells showed increased adherence to the substratum and exhibited
morphological changes which included flattening of cell bodies and the enhanced elaboration of thick processes. Thus, bFGF appears to act as a mitogen and as an initiator of differentiation. This heterogeneity of response does not appear to be related to the heterogeneity of CHP100 cells with regard to I- and N-type morphology, since subclones with differing proportions of I- and N-type cells responded to FGF similarly. Birren and Anderson (5) noted a similar combination of mitogenic and differentiative responses in their v-myc-immortalized sympathoadrenal progenitor cell line treated with bFGF. Another characteristic shared between these cells and CHP100 cells is the increase of p75NGFR immunoreactivity induced by culture with bFGF. These findings are consistent with a dual role of bFGF in sympathetic neuronal development wherein bFGF alternatively promotes proliferation or neuronal differentiation.

CHP100 cells did not appear to be responsive to NGF with regard to cell morphology, cell survival, or regulation of p75NGFR expression, even after expression of p75NGFR had been stimulated by exposure to bFGF. In this respect, CHP100 cells do not accurately reflect the postulated behavior of cells of the sympathetic neuronal lineage in vivo. This insensitivity to NGF apparently results from the absence of p140F, since we were unable to detect p140F mRNA in these cells by Northern blot analysis (data not shown). In this regard, CHP100 cells differ from most sympathetic neurons which have abundant trk mRNA (in rats). However, a subpopulation of sympathetic neurons express trkB and therefore are presumably responsive to BDNF rather than NGF (34, 49a). We have not determined whether CHP100 cells resemble this population of sympathetic neurons, and if so, whether expression of p75NGFR functions with regard to response to BDNF.

Regulation of NGFR promoter by bFGF in CHP100 cells. Northern blot analysis (Fig. 5) showed that enhanced p75NGFR immunoreactivity in bFGF-treated CHP100 cells is attributable, at least in part, to increased steady-state levels of p75NGFR mRNA. The ability of bFGF to enhance expression of the NGFR promoter-CAT fusion gene pRBLCAT in CHP100 cells suggests that the stimulation of p75NGFR expression by bFGF is due, at least in part, to increased NGFR gene transcription. However, further detailed analyses will be required to assess whether regulation of p75NGFR expression is entirely at the transcriptional level.

It appears that regulation of CAT gene expression by 5'-flanking sequences of the NGFR gene mimics regulation of the endogenous NGFR gene during bFGF-induced differentiation. Earlier studies of Sehgal et al. (52) demonstrated that sequences extending 1.2 kb upstream of the NGFR gene transcriptional start site contained a promoter activity which was extremely active in fibroblastic cells although these sequences did not express their endogenous NGFR gene. Clearly then, these 5'-flanking elements are not sufficient to yield entirely correct regulation of NGFR gene expression. Our preliminary results (unpublished) suggest that longer 5'-flanking segments, such as those employed in the present study, also yield inappropriate levels of promoter activity in fibroblastic cells. Nevertheless, our results show that these promoter sequences do contain regulatory elements which are able to confer some aspects of correct regulation, since bFGF enhances expression of the promoter-reporter gene construct in a manner similar to that of the endogenous NGFR gene in CHP100 cells and in a manner analogous to that which has been postulated to occur in vivo (5). We have recently observed that implantation of bFGF-containing polymer pellets into rabbit corneas induces p75NGFR expression in adjacent posterior corneal epithelial cells (48a). This result suggests that the gene regulatory elements responsible for bFGF induction of p75NGFR expression in neuronal cells may also function in some nonneuronal cells.

We believe we have defined a system which will be suitable for characterizing the cis regulatory element(s) of the NGF receptor gene and corresponding transcriptional factors involved in induction of NGF gene expression by bFGF (or related factors) during neuronal differentiation. Such investigations are likely to provide valuable insights concerning the molecular mechanisms underlying neural development.

ACKNOWLEDGMENTS

We thank Moses Chao for providing the human NGFR genomic clone 17A2. We also thank Chiron Corporation for the gift of human bFGF.

This work was supported by Public Health Service grants NS23343 (to M.B.) and NS08716 (to K.L.D.), and by a grant from Sumitomo Pharmaceutical Company, Japan (to M.B.).

REFERENCES

NGF RECEPTOR INDUCTION BY FGF


49. Santos, P., and M. Bothwell. Unpublished data.


51. Schecterson, L. C., and M. Bothwell. Submitted for publication.


