Regulation of Expression of the Neuronal POU Protein Oct-2 by Nerve Growth Factor*

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POU proteins are a class of homeobox-containing transcription factors that regulate tissue-specific gene expression and influence cell differentiation and function. We have investigated the possible role of such factors in mediating the actions of nerve growth factor (NGF) on sensory neurons. NGF has been found to have differential effects on the levels of three POU protein transcription factors that are expressed in adult rat sensory neurons. A sensory neuron octamer-binding protein with the properties of the transcription factor Oct-2 is up-regulated 3–4-fold by NGF, as measured by mobility shift assays using nuclear extracts from adult rat dorsal root ganglion neurons grown in the presence or absence of NGF. Quantitation of Oct-2 mRNA by polymerase chain reaction amplification of RNA from such cells shows a parallel increase in Oct-2 mRNA levels. In contrast, the levels of mRNA encoding the ubiquitous POU protein Oct-1 or the neuron-specific POU protein Brn-3, also present in sensory neurons, are unaffected by NGF. These observations suggest a role for Oct-2 in mediating transcriptional effects induced by NGF. In particular, as Oct-2 is known to inhibit herpes simplex virus immediate-early gene expression in neuronal cells, these findings provide a mechanism for the known action of NGF in the maintenance of latent herpes virus infections in sensory neurons.

Nerve growth factor (NGF) is one of a family of neurotrophic factors that, among other actions, promote the survival of developing peripheral sensory and sympathetic neurons. Neuronal survival in the developing peripheral nervous system has been demonstrated to depend in part upon the presence of limiting amounts of NGF (Barde, 1989). A family of related factors (BDNF, NT3, NT4/5) with remarkably conserved protein sequences between species that show subtly different profiles of cellular specificity as survival factors in vitro have recently been identified by molecular cloning (Mainsonpiere et al., 1990; Berkemeier et al., 1991; Hallbook et al., 1991). A high affinity receptor for NGF has also been identified and been demonstrated to be a transmembrane protein with intrinsic tyrosine kinase activity (TrkA) (Kaplan et al., 1991; Klein et al., 1991). Structurally related transmembrane tyrosine kinases (TrkB, TrkC) are good candidates as cognate receptors for the other neurotrophins so far identified (Squinto et al., 1991; Lamballe et al., 1991). Despite these advances, and the cataloguing of NGF-induced second messenger changes and effects on gene expression in susceptible cells (Chao, 1992), the physiological role of neurotrophic factors in the mature nervous system and the mechanism of their action are poorly understood. Artificial elevation of NGF levels has been shown to alter the phenotype of nociceptive sensory neurons that play a role in the neuronal component of inflammatory responses, and effects on the expression of various neuropeptides and ion channels at the mRNA level in these cells have been described (Lindsay and Harmer, 1988). It therefore seems likely that many actions of NGF are mediated through effects on transcriptional regulation. Consistent with this, a number of NGF-regulated transcription factors have been identified in the phaeochromocytoma-derived PC12 cell line by differential screening methods (Milbrandt, 1987; Oppenheim, 1991).

We have used primary cultures of peripheral neurons as an in vitro system to investigate the action of NGF on the POU protein class of transcription factors that appears to have particular importance in tissue-specific gene regulation (Herr et al., 1988; He et al., 1989). Adult rat sensory neurons are known to express a number of such proteins, including Oct-1, Oct-2, Brn-3, and a number of Brn-3-related factors, the partial sequences of which have been identified by PCR (Latchman et al., 1992). Two such factors, Brn-3 and Oct-2, are of particular interest because of their restricted cellular distribution. The octamer-binding protein Oct-2, a factor first identified as a B cell immunoglobulin-specific transcription factor has subsequently been found in a number of types of neurons including sensory neurons (He et al., 1989; Lillycrop et al., 1991). The brain-derived putative transcription factor Brn-3 is additionally interesting because of its close structural similarity to a protein that determines the developmental fate of some sensory neurons in Caenorhabditis elegans, first identified in the Unc-86 mutant (Herr et al., 1988). We therefore analyzed the effects of NGF on the levels of mRNA encoding the neuron-specific class IV POU protein Brn-3 and the tissue-specific octamer binding protein Oct-2 as well as Oct-1, a ubiquitously expressed POU protein, that is known to play a critical role in regulating the expression of a number of cellular and viral genes by binding to the octamer sequence ATGCAAAT in their promoters (Falkner et al., 1986). Adult rat sensory neurons, unlike their neonatal counterparts,
can survive in culture without NGF (Lindsay et al., 1988; Winter et al., 1988). We were therefore able to examine the effect of depleting NGF on the expression of transcription factors that have been identified in adult rat sensory neurons without compromising the viability of the cultures. We report here that studies using quantitative polymerase chain reaction amplification of sensory neuron RNA demonstrate specific up-regulation of Oct-2 mRNA levels by NGF in adult rat sensory neurons, together with an increase in Oct-2 protein levels measured in mobility shift assays. This effect is specific for Oct-2, as the POU proteins Brn-3 and Oct-1 as well as other non-POU transcription factors such as TFIIIC are unaffected by alterations in the levels of NGF.

MATERIALS AND METHODS

Cell Culture—Dorsal root ganglia from all spinal levels of adult male Sprague-Dawley rats were dissected aseptically and collected in Ham’s F14 medium supplemented with 170 µg/liter sodium bicarbonate, 1 mM glutamine, 100 µg/ml penicillin, and 100 units/ml streptomycin. Ganglia were digested with 0.15% collagenase (Boehringer) and mechanically dissociated through a fine polished Pasteur pipette. Cells were then preplated overnight on polyornithine-coated Petri dishes. After 24 h, lightly adherent neurons were removed from the surface of the medium and replated on 13-mm glass coverslip previously coated with polyornithine and laminin (Bethesda Research Laboratories) at a density of 20,000 neurons per coverslip. Cultures were then supplemented with 0.1 µg/ml 2.5 S NGF prepared from mouse salivary glands (Suda et al., 1978) or grown in the absence of NGF and presence of neutralizing sheep anti-NGF antiserum at concentrations capable of neutralizing 0.2 µg/ml NGF to block any endogenously synthesized NGF produced by nonneuronal cells in the cultures. An equivalent concentration of normal sheep serum was added to cultures grown in the presence of NGF. Cytosine arabinoside (10 µM) was included in the medium for the first 2 days to kill dividing cells, then removed, and 5 days after plating either total RNA was extracted from the cells by a guanidine chloride/phenol extraction procedure (Chomczynski and Sacchi, 1987) or nuclear extracts were prepared for mobility shift assays.

cDNA Probe Synthesis and Northern Blot Analysis—A neonatal rat dorsal root ganglion (DRG)d cDNA library constructed in λ Zap11 (the kind gift of Dr. J. Boulter) was plated at 1–2 × 10⁸ plaque-forming units per 132-mm-square plate and plaques grown for 7 h at 42 °C before binding to Hybond N membranes (Amersham). The filters were denatured and neutralized and UV-irradiated. The filters were prehybridized for 5 h in 6 × SSC, 5 × Denhardt’s solution 100 µg/ml salmon sperm DNA, 5 mM EDTA and 0.2% SDS at 42 °C in the same solution containing 10⁶ cpm/ml of a denatured oligo-labeled Brn-3 probe comprising the POU and homeobox domain. After hybridization, the filters were washed at room temperature for 20 min in 0.2 × SSC and 3 min in 0.2 × SSC/0.2% SDS at 68 °C. Plaque-purified positive clones were sequenced using Sequenase II (United States Biochemical). Neonatal rat DRG total RNA (20 µg) or poly(A)+ RNA (2 µg) was fractionated on 1% agarose/formaldehyde gels and blotted on to Hybond N filters. After 30 min at 4 °C, the nuclear extracts were microfuged for 15 min at 4 °C, and supernatants were stored in 20-µl aliquots. 10 fmol of labeled probe was added to 1 µl of nuclear extract in a total volume of 20 µl of Hepes, pH 7.9, 5 mM MgCl₂, 50 mM KCl, 0.5 mM DTT, 4% Ficoll, 2 µg of poly(dI-dC) (Pharmacia LKB Biotechnology Inc.) at 4 °C for 40 min, after which DNA protein complexes were separated by electrophoresis on 4% polyacrylamide gels run for 2-3 h at 150 V at 4 °C in 2.5 mM Tris, 2.5 mM boric acid, 0.05 mM EDTA, pH 8.3.

Quantitative PCR—In preliminary experiments reverse transcription PCR amplification were carried out using the polymerase chain reaction amplification from normal rat (L27) 130-µl cDNA reactions (the kind gift of Dr. J. Boulter) was plated at 1-2 × 10⁶ cells. A 10-ng aliquot of this RNA was added to mRNA samples from DRG controls and 3′ region of Brn-3 (30 s at 95 °C, 45 s at 57 °C, 1 min at 72 °C) using primer sets from a rat (L27) 130-µl cDNA reaction (Lillycrop et al., 1991). Amplifications were carried out for 25 cycles of amplification as previously described (Lillycrop et al., 1991). Polymerase chain reaction of the Oct-2 mRNA used a control internal template prepared by the transcription of a human Oct-2 cDNA (Clerc et al., 1988) using T7 polymerase. A 10-ng aliquot of this RNA was added to mRNA samples from DRG cells cultured in the presence (+) or absence (−) of NGF. Following amplification with Oct-2-specific primers, the product from the control human Oct-2 RNA and from the rat Oct-2 mRNA in the sample was distinguished by restriction enzyme digestion with BglII which cuts only the human Oct-2 PCR product to product fragments of 108 and 108 base pairs, while not affecting the rat Oct-2 PCR amplification. PCR amplifications from the 3′ region of Brn-3 (30 s at 95 °C, 45 s at 57 °C, 1 min at 72 °C) using primer sets from a rat oligo-labeled Brn-3 probe (He et al., 1991) was washed at 42 °C. After centrifugation X at 1000 g for 10 mins, the cell pellet was resuspended in 3 volumes of 4% Ficoll, 2 µg of poly(dI-dC) (Pharmacia LKB Biotechnology Inc.) at 4 °C for 10 mins, the cell pellet was resuspended in 3 volumes of the above buffer to which Nonidet P-40 (0.05%) was added. The cells were disrupted in a Potter homogenizer at 20 strokes, isolated by centrifugation at 1000 g X 10 mins. The nuclear pellet was then resuspended in 100 µl of buffer C (25 µl of 2 M Hepes, pH 7.9, 1.25 µl of 50% glycerol, 3.75 µl of MgCl₂, 1.25 µl of 1 M DTT, 12.5 µl of 100 mM phenylmethylsulfonyl fluoride in a total volume of 2.5 ml), and 4 M NaCl added to a final concentration of 300 mM. After 30 min at 4 °C, the nuclear extracts were microfuged for 15 min at 4 °C, and supernatants were stored in 20-µl aliquots. 10 fmol of labeled probe was added to 1 µl of nuclear extract in a total volume of 20 µl of Hepes, pH 7.9, 5 mM MgCl₂, 60 mM KCl, 0.5 mM DTT, 4% Ficoll, 2 µg of poly(dI-dC) (Pharmacia LKB Biotechnology Inc.) at 4 °C for 40 min, after which DNA protein complexes were separated by electrophoresis on 4% polyacrylamide gels run for 2-3 h at 150 V at 4 °C in 2.5 mM Tris, 2.5 mM boric acid, 0.05 mM EDTA, pH 8.3.

RESULTS AND DISCUSSION

Actions of NGF on POU Protein mRNA Levels—RNA was extracted from adult rat sensory neurons grown in the presence or absence of NGF, with anti-NGF antiserum added to NGF-free cultures to neutralize any NGF released from nonneuronal cells. Because of the limited amounts of material available from the DRG cultures and the low abundance of transcription factor mRNA, the mRNAs were quantitated using PCR with oligonucleotide primers specific to each of the POU domain sequences of the Oct-1, Oct-2, or Brn-3 mRNAs. Because a variety of Brn-3-like clones with similar but not identical POU domain sequences have been identified in neuronal cells by PCR amplification (Latchman et al., 1989), we screened a rat DRG library with the originally described Brn-3 POU domain as a probe (He et al., 1989) and isolated and sequenced overlapping clones, in order to identify PCR primers uniquely directed at Brn-3 itself. Those clones that contained partial sequence with 100% homology to the original clone were used to probe Northern blots of DRG RNA. Both the original clone and a 3′ overlapping clone hybridized to a 4-kb mRNA (Fig. 1). We therefore used additional primers across the 3′-untranslated region of Brn-3 sequence derived from the new clone, as well as primers for the POU domain of Brn-3 to be certain that we were measuring Brn-3 mRNA levels. As an external control, primers specific for a constitutively expressed control mRNA encoding the ribosomal protein L27 were also used (Lebeau et al., 1991). In each experiment, the identity of the PCR product was
confirmed both by digestion with appropriate restriction enzymes and by hybridization with Oct-1, Oct-2, or Brn-3-specific cDNA clones. To ensure that the PCR was quantitative, preliminary experiments were carried out by varying the amounts of mRNA and cycle numbers to identify conditions in which the signal obtained was linearly related to the amount of input RNA. In addition, in some experiments we included equal amounts of a control human Oct-2 RNA template prepared by the transcription of a human Oct-2 plasmid whose PCR product could be distinguished from that of rat amounts of input RNA. In some experiments we isolated from adult sensory neurons grown in the presence of NGF, and DNA mobility shift assays using the octamer binding site ATGCTAATGATAT but less strongly to an oligonucleotide containing the binding site for the SP1 unrelated oligonucleotide containing the binding site for the SP1 sequence specificity for different octamer oligonucleotides which we have previously shown distinguish neuronal Oct-2 from the B cell form of Oct-2 and other octamer binding proteins (Dent et al., 1991). Thus in competition experiments the protein bound strongly to the octamer oligonucleotide ATGCTAATGATAT but less strongly to an oligonucleotide.
ATGCTAATGAGAT containing a single base change which reduces the binding of neuronal Oct-2 while not affecting binding of B cell Oct-2 or Oct-1 (Fig. 3C). The up-regulation of neuronal Oct-2 in the NGF-treated cells was specific to this factor, as the multiple complexes produced by association of the ubiquitous polymerase III-associated transcription factor TFIIIC with its binding site were not diminished by removal of NGF (Fig. 3B). Similarly, no increase was observed in NGF-treated cells of the levels of a high mobility octamer-binding complex (arrowed in Fig. 3) which was specifically competed only by octamer oligonucleotide (Fig. 3C) and is therefore likely to be formed by one of the low molecular weight octamer-binding proteins identified by others in neuronal cell lines (Scholer et al., 1989). Moreover, on longer autoradiographic exposure, a low mobility octamer-binding complex identical in size to that produced by binding of Oct-1 was observed at equal levels in both the NGF-treated and untreated cells (data not shown). These results therefore demonstrate that the levels of Oct-2 protein are regulated by NGF at the mRNA level, while the related transcription factor Oct-1 remains unaffected.

NGF is known to up-regulate the mRNA for a number of transcription factors in PC12 cells, such as the NGFI-A zinc-finger protein that is also induced in a variety of neuronal and non-neuronal cell types through activation of SRE-related sequences (Changelian et al., 1989). Brn-3, found at high levels in developing and adult DRG neurons was first isolated from rat brain by PCR and found to have structural similarities to the Unc-86 protein that determines neuronal cell fate in C. elegans. As sensory neurons are known to depend upon NGF for survival during development, it was of particular interest to see if Brn-3 mRNA levels were up-regulated by NGF. The studies on adult neurons in culture here provide no evidence that this is the case, but the possibility that NGF regulates Brn-3 levels during development, which is not amenable to analysis in this experimental system remains an open question. DRG neurons also contain a number of POU proteins that are related to Brn-3 (Latchman et al., 1992). In this study we have used PCR primers derived from the POU domain of Brn-3, as well as primers derived from an overlapping 3’ clone that hybridize to a 4-kb Brn-3 transcript in DRG RNA. There remains the possibility either that related Brn-3-like proteins are transcriptionally regulated by NGF, or that posttranscriptional regulation occurs; further sequence information about this family of proteins is required to assess these possibilities. The recent demonstration of differentially spliced homologous class IV POU proteins from Drosophila (I-POU and It-POU) with opposing effects on transcription (Trecay et al., 1992) suggest that an analogous situation could exist for Brn-3 like-proteins. The role played by the Oct-2 protein in B cells in positively regulating the expression of immunoglobulin genes (Scheider, 1987) suggests that neuronal Oct-2 may play a similarly significant role in gene regulation in sensory neurons and their response to NGF. Although the cellular genes regulated by Oct-2 in sensory neurons remain to be identified, one obvious candidate is the gene encoding the neuropeptide CGRP which contains two octamer motifs in its promoter (Broad et al., 1989) and whose mRNA increases in abundance in adult sensory neurons treated with NGF (Lindsay and Harmer, 1989). It is probable however, that Oct-2 may act primarily to inhibit rather than activate gene expression in neuronal cells. A number of differently spliced variants of Oct-2 have been identified (Wirth et al., 1991), and there is evidence that the forms expressed in neuronal cells may differ in their activity from those expressed in B cells (Dent et al., 1991). Thus unlike B cell Oct-2, neuronal Oct-2 has been shown to be ineffective at activating reporter constructs containing an octamer motif and can interfere with the activation of such constructs by Oct-1 (Dent et al., 1991), suggesting that Oct-2 acts as an inhibitor of octamer-mediated gene regulation. This potential inhibitory role of neuronal Oct-2 is of particular interest with respect to the mechanism of infection of these cells by herpes simplex virus (HSV). Sensory neurons support a latent form of asymptomatic HSV infection in an NGF-dependent manner (Wilcox and Johnson, 1988). Depleting the supply of nerve growth factor to the cells results in the re-expression of virus which lytically infects cells innervated by the infected neuron. Evidence for the existence of an NGF-regulated repressor of viral reactivation has been obtained in rat, monkey, and human sensory neurons (Wilcox et al., 1990). The failure of the HSV lytic cycle in neuronal cells with the consequent production of a latent infection has been shown to be dependent on the weak activity of HSV immediate-early promoters in sensory neurons, due to the presence of an inhibitory factor that binds to the viral regulatory octamer-related motif TAATGARAT. This inhibitory factor has been identified as neuronal Oct-2 by mobility shift assays, and elevation of the levels of Oct-2 in neuronal cell lines has been shown to exert an inhibitory action on immediate-early gene expression using reporter gene constructs (Kemp et al., 1990; Lillycrop et al., 1991). This inhibitory action reflects the competition for the viral octamer binding site by Oct-1 which forms a productive trans-activating transcriptional complex with the HSV virion protein Vmw65 and Oct-2 which does not (Gerster and Roeder, 1988). The demonstration that NGF up-regulates Oct-2 without altering the levels of Oct-1 thus provides a mechanism for the repression of HSV immediate-early gene expression and maintenance of latent infection by NGF and is consistent with the view that Oct-2 is the inhibitor of herpes virus expression. Failure of retrograde transport of NGF caused by tissue damage would lead to a fall in Oct-2 levels with a consequent activation of HSV immediate-early gene transcription and production of virus. It will therefore be important to determine the precise molecular structure of sensory neuron Oct-2 and to test the actions on Oct-2 mRNA levels of other neurotrophic factors such as NT3 and BDNF as well as mediators such as glucocorticoids that play important homeostatic roles and may also influence HSV expression.

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