Altered protein binding to the octamer motif appears to be an early event in programmed neuronal cell death
(apoptosis/nerve growth factor/DNA–protein binding/PC12 cells/electrophoretic mobility-shift assay)

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ABSTRACT
Electrophoretic mobility-shift assays were used to characterize binding of nuclear proteins to consensus sequences for Sp1, E2F, octamer, and cAMP responsive enhancer element (CRE) during neuronal death in vitro after removal of nerve growth factor (NGF). Molecular events occurring prior to cell death in terminally differentiated PC12 cells could be divided into three phases: (i) within 2 hr of removing NGF, binding to the octamer sequence decreased; (ii) after 5–7 hr an increase in binding to CRE occurred; and (iii) after 14 hr (at which point 50% of the cells are committed to die) a decrease in binding to the Sp1 sequence occurred. Assays performed with extracts from sympathetic ganglia indicated that changes in binding to CRE and octamer motifs also occurred during the period of developmental cell death in vivo. Double-stranded oligonucleotides were delivered to neurons to act as dominant negative “promoters” unable to couple to transcriptional events but capable of binding and sequestering transcription factors. Double-stranded but not single-stranded octamer oligonucleotides increased cell death of primary cultures of sympathetic neurons. Most of the induced neuronal cell death could be blocked with NGF, which is consistent with oligonucleotides activating an endogenous death program rather than having a nonspecific toxic effect. Other double-stranded oligonucleotides as well as a mutant octamer oligonucleotide had little or no effect on cell death. These data are consistent with the hypothesis that cell death results from a cascade of cellular and molecular events and that an early event in programmed neuronal cell death is a decrease in binding of transcription factor(s) to octamer motif sequences.

In the vertebrate nervous system approximately half the neurons generated in many neuronal populations die over a relatively short period of time, typically during the period of synaptogenesis (1). The emerging view is that developing neurons compete for a limited amount of target-derived trophic factors, the function of which is to inhibit the activation of endogenous cell death genes (2–4). Neurons that die during development and possibly later in life during aging and in neurodegenerative diseases such as Alzheimer and amyotrophic lateral sclerosis may do so because they are unable to obtain sufficient quantities of trophic factors to block an endogenous cell death program. A fundamental knowledge of genes that control cell death (5) and the molecular cascade of events resulting in cell death is necessary for understanding this important regulatory event.

The present study was initiated to determine whether changes in protein binding to consensus DNA sequences for known transcription factors occur during early phases of neuronal cell death. The data support the idea that a cascade of molecular events occurs after removing nerve growth factor (NGF) and demonstrate that double-stranded oligonucleotides can modify cellular function, presumably by competing with promoter sequences for endogenous transcription factors. Data suggest that changes in protein binding to octamer sequences are an early event in neuronal cell death.

MATERIALS AND METHODS

Cell Culture. PC12 cells were cultured and terminally differentiated as described (6). Experiments were initiated by removing NGF-containing medium and replacing it with serum-free medium containing N2 components (7) and NGF IgG (75 μg/ml). Sympathetic neurons were isolated from the superior cervical ganglia of postnatal day 1–2 rats and cultured in serum-free Dulbecco’s modified Eagle’s medium (DMEM)/F12 medium (1:1) containing N2 components on a laminin substrate.

Oligonucleotides and Electrophoretic Mobility-Shift Assays. Oligonucleotides used in mobility-shift assays and for treating cultured neurons include the following: octamer, 5′-TGGTAAATTGCTTTCAAA-3′; mutant octamer, 5′-TGGGTAATGCGCATTTCAAA-3′; herpes simplex virus (HSV) octamer, 5′-ATGCTAATGATATCGT-3′; Sp1, 5′-CTAAGATCTGCGGGGCTCTTAG-3′; cAMP responsive enhancer element (CRE), 5′-GATCCAATTGACGTCAAGATC-3′; E2F, 5′-AGTTTCCGCTTAAATTGAGAAGGGCGCAACTG-3′; mutant E2F, 5′-AGTTTCTCGACCTTAAGATTGAGAAGGGCGCAACTG-3′. Sequences in boldface denote consensus sequences, and underlined bases represent bases altered to produce mutant oligonucleotides. Oligonucleotides were annealed to complementary sequences, purified on a 20% polyacrylamide gel, and used as double-stranded oligonucleotides. Nuclear extracts were isolated and binding reactions were performed according to standard procedures (8). Whole cell extracts were prepared according to Scholer et al. (9) and stored at −80°C. Specific DNA–protein interactions were defined by including a 50- to 100-fold excess of unlabeled double-stranded oligonucleotide or by performing reactions with mutant double-stranded oligonucleotides.

Isolating Cell Fractions. A modification of the procedure used to prepare nuclear extracts was used to generate membrane, cytoplasmic, and nuclear fractions. In short, cells were lysed in hypotonic buffer using a Dounce homogenizer, and the nuclear fraction was isolated after centrifugation at 1000 × g. The supernatant from the nuclear fraction was centrifuged at 30,000 × g to generate crude cytoplasmic (supernatant) and membrane (pellet) fractions. The pellet from the nuclear fraction was suspended in a 100-fold excess of isotonic buffer and centrifuged at 1000 × g; this procedure was repeated twice to decrease cytoplasmic contamination in the nuclear fraction.

Abbreviations: NGF, nerve growth factor; CRE, cAMP responsive enhancer element; HSV-1, herpes simplex virus 1.

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Treating Neurons with Oligonucleotides. Sympathetic neurons from the superior cervical ganglia of 1- to 2-day-old rat pups were mechanically dissociated, triturated vigorously, and immediately plated onto laminin-coated 96-well plates containing double-stranded oligonucleotides in serum-free DMEM/F12 medium. This method of trituration loading increases uptake of both oligonucleotides and proteins into cells (10-12). After 14-16 hr of culturing, counts of viable neurons were made in a 0.05-cm² field in the center of each well; strong correlations exist between cell counts and cell viability determined with the tetrazolium dye MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (r = 0.94) or by release of lactate dehydrogenase into the medium (r = 0.97; see ref. 6).

RESULTS

Under appropriate culture conditions, PC12 pheochromocytoma cells treated with NGF for 14 days become postmitotic and dependent on NGF for survival (6). After removal of NGF, RNA and protein synthesis are required for cell death (Fig. 1 A-C; see also refs. 6, 13, and 14). Endonuclease activation with subsequent DNA laddering into nucleosomal fragments is characteristic of apoptotic cell death (15-17). An endonuclease is activated in PC12 cells after removal of NGF (Fig. 1D); however, DNA fragmentation is not responsible for death of these cells (6). After removal of NGF, 50% of the cells are committed to die (defined as the point when addition of NGF does not block subsequent death) after 12 hr and initial signs of degeneration are present after 14-20 hr (6).

The possibility that changes in binding of transcription factors to consensus DNA sequences occur soon after removing NGF but before cell death was investigated by performing electrophoretic mobility-shift assays with nuclear proteins isolated at different times after removal of NGF. Sp1 is a constitutive transcription factor that binds to G+C-rich regions in promoters of a large number of genes (18). Nuclear proteins from actively proliferating undifferentiated PC12 cells exhibit three major complexes when incubated with a double-stranded oligonucleotide containing the Sp1 motif (Fig. 2A). Nuclear proteins from NGF-treated cells exhibit the same three complexes; however, the intensity of the bands is considerably less than in proliferating PC12 cells (Fig. 2A). After removal of NGF, no consistent changes in DNA-protein interactions occur at 2 or 7 hr (an inconsistent increase in binding is occasionally seen after 7 hr); however, after 14 hr a decrease in protein binding to the Sp1 element occurs (Fig. 2A; an overall decrease in the quantity of total nuclear proteins is also first detected after 14 hr). A 50-fold excess of unlabeled double-stranded oligonucleotide inhibits binding, indicating selectivity for the Sp1 element.

CRE is an element present in genes regulated by cAMP (19). Binding to CRE is greater in undifferentiated prolifer-
ating cells than in differentiated cells (Fig. 2B). No change in protein binding to CRE is seen 2 hr after removing NGF, although by 5-7 hr an increase in protein binding to CRE is observed (Fig. 2B; by densitometry, a 95% increase occurs at 7 hr compared to controls). Increased binding to CRE is maintained at 14 hr (by densitometry, a 90% increase compared to controls), which is the time when 50-60% of the cells are irreversibly committed to die.

The octamer motif is an 8-nucleotide cis-acting element that binds members of the POU domain family of transcription factors (20-22). Whereas decreased binding to CRE and Sp1 elements is observed with nuclear proteins isolated from differentiated PC12 cells compared to undifferentiated proliferating cells, binding to the octamer motif is more extensive in differentiated PC12 cells (Fig. 2C). Differentiated PC12 cells exhibit five different DNA–protein binding complexes compared to three complexes in proliferating PC12 cells. Within 2 hr of removing NGF, decreases in binding to the octamer sequence occur (Fig. 2C; by densitometry a 40-50% decrease in intensity for four of the five bands), and a progressive decrease in binding is seen over the next 12 hr (by 14 hr an 80-90% decrease in intensity for all bands is observed). Very little binding occurs with a mutant octamer sequence (Fig. 2C).

E2F is a transcription factor that binds to cis-acting elements in promoters of genes involved in cell proliferation such as c-myc and is negatively regulated by the retinoblastoma protein (23-25). Binding to the E2F motif is characterized by one major and one minor DNA–protein complex in proliferating PC12 cells (Fig. 2D). The major DNA–protein complex does not form with a mutant E2F oligonucleotide, whereas the minor DNA–protein complex does form with the mutant E2F oligonucleotide (Fig. 2D). Both complexes are inhibited by a 50-fold excess of unlabeled oligonucleotide (data not shown). Only the major DNA complex is present in NGF-treated cells, and this complex exhibits little change at 2, 7, or 14 hr after removal of NGF (Fig. 2D).

Removal of NGF from differentiated PC12 cells results in early changes in binding of nuclear proteins to octamer and CRE motifs that occur several hours before cells become committed to die. Although changes in binding to these elements may be involved in events leading up to cell death, a stronger case for a role in cell death could be made if changes in binding also occur during programmed cell death in vivo. There are no homogeneous neuronal populations in vivo in which a large fraction of cells die over a short period of time; however, about one-third of the neurons in sympathetic ganglia die over a 3- to 4-day period during development (26). Sympathetic neurons also require NGF for survival and PC12 cells most closely resemble sympathetic neurons once they are differentiated with NGF. Therefore, electrophoretic mobility-shift assays were performed with whole cell extracts prepared from sympathetic ganglia before (postnatal day 2), during (postnatal day 5), and after (postnatal day 7) the period of developmental cell death. Binding to Sp1 and E2F motifs was similar before, during, and after the period of cell death (Fig. 3 A and D), while binding to CRE and octamer motifs was altered during the period of cell death. Binding of whole cell extracts to CRE decreases during the period of programmed cell death but is similar before and after the period of cell death (Fig. 3B; by densitometry a 70% decrease in the upper band(s) occurs during the period of cell death—based on our unpublished studies with mutant CRE oligonucleotides, the top bands are selective for CRE (see also lower shifted band is not). Electrophoretic mobility-shift assays with the octamer motif indicate that one DNA–protein complex increases in intensity and an additional complex is present during the period of programmed cell death (Fig. 3C). Excess unlabeled double-stranded oligonucleotides inhibit binding (Fig. 3), indicating

![Fig. 3. Binding of consensus DNA motifs to whole cell extracts from sympathetic ganglia before, during, and after the period of developmental cell death in vivo. Whole cell extracts (15 µg) were incubated with double-stranded 32P-labeled oligonucleotides representing consensus sequences for Sp1(A), CRE(B), octamer(C), and E2F(D) followed by electrophoresis and autoradiography. FP, free probe (32P-labeled oligonucleotide in the absence of cell extracts); +, 50-fold excess of unlabeled oligonucleotide included in binding reaction mixture to define specific binding; D2, D5, and D7, postnatal day 2, day 5, and day 7 ganglia extracts (representing times before, during, and after the period of developmental cell death). Similar patterns were observed in two separate experiments.](image-url)
in the absence of NGF during the first 24 hr); therefore, effects of oligonucleotides on neuronal survival can be determined in the absence or presence of NGF. Uptake of double-stranded oligonucleotides is more rapid and reaches a higher level when uptake is by trituration loading (10–12) rather than by simple uptake (preliminary experiments indicated that 3.6% of total oligonucleotides are taken up following trituration loading vs. 2.8% by simple uptake); therefore, experiments were carried out with trituration loading of double-stranded oligonucleotides. Incubating cells with double-stranded oligonucleotide containing a trace amount of 32P-labeled oligonucleotide followed by cell fractionation indicates that 28% of cellular radioactivity is associated with the membrane fraction, 68% is associated with the cytoplasmic fraction, and 4% is associated with the nuclear fraction (two additional washes of the nuclear fraction were performed to decrease contamination by cytoplasmic components). The majority of the radioactivity in the nuclear fraction is present as double-stranded oligonucleotide rather than degraded oligonucleotides or single-stranded oligonucleotides [Fig. 4A (Inset); a complex between 32P-labeled double-stranded oligonucleotide and nuclear proteins is not observed because of the 100-fold excess of unlabeled double-stranded oligonucleotide present]. Enhanced cell death of sympathetic neurons occurs in cultures treated with 1.5 μM octamer and HSV octamer (a modified octamer sequence found in promoters of HSV-1 immediate-early genes) double-stranded oligonucleotides compared to cultures treated with CRE, E2F, and Sp1 oligonucleotides (Fig. 4B). NGF blocks a large amount of the cell death (Fig. 4D), which is consistent with oligonucleotides activating an endogenous cell death program rather than having nonspecific toxic effects. It also suggests that NGF blocks cell death "downstream" from changes in binding to the octamer motif or, alternatively, that NGF increases octamer binding proteins (see ref. 31). A large number of studies have shown that antisense single-stranded oligonucleotides can decrease mRNA and/or protein synthesis (for review, see ref. 32); therefore, the effects of octamer oligonucleotides might result from dissociation of double-stranded oligonucleotides into single strands followed by alterations in RNA and/or protein synthesis. If this were the case, then single-stranded octamer oligonucleotides would be expected to increase cell death. Even at 3 μM, neither 3' → 5' nor 5' → 3' single-stranded octamer oligonucleotides increase cell death (Fig. 4C). In addition, a double-stranded mutant octamer oligonucleotide that does not bind DNA (see Fig. 2C) does not affect neuronal cell death (Fig. 4C).

**DISCUSSION**

Data in the present study are consistent with the idea that a cascade of molecular events is responsible for neuronal cell death after loss of trophic support. The earliest change detected by using gel-shift assays is a decrease in nuclear protein binding to the octamer motif, which occurs 1–2 hr after removing NGF. Proteins binding to the octamer motif are members of the POU family of homeodomain-containing transcription factors (20, 21). Oct-1 is a transcription factor found in most cell types, while other members of the family are highly restricted both temporally and spatially (20, 22, 33–35). Oct-2 was originally considered a lymphocyte-specific transcription factor involved in immunoglobulin expression; however, six isoforms of Oct-2 have been identified and many of these are present in tissues other than lymphocytes (21, 36). In neurons, Oct-2 is induced by NGF (31) and is required to maintain HSV-1 in a latent state by acting as a repressor of HSV-1 immediate-early genes (37, 38).

After removal of NGF from cells in vitro, binding to CRE increases and binding to the octamer motif decreases, whereas during the period of cell death in vivo binding to CRE decreases and binding to the octamer element increases. There are three possible explanations for differences in

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**Fig. 4.** Uptake and effect of double-stranded oligonucleotides on survival of sympathetic neurons. (A) Uptake of 32P-labeled double-stranded octamer oligonucleotide into cells as a function of time. (Inset) Autoradiogram of a 20% polyacrylamide gel containing double-stranded (Ds) and single-stranded (Ss) oligonucleotide standards (lane 1) and the twice-washed nuclear fraction showing the presence of double-stranded oligonucleotide in the nucleus after 4 hr (lane 2). Sympathetic neurons were triturred and grown in the presence of 1.5 μM double-stranded oligonucleotide for 14 hr on a laminin substrate in the absence (B) or presence (D) of NGF and cell survival was determined. Oct, octamer sequence; HSV, modified octamer sequence present in HSV-1 immediate-early genes. (C) Sympathetic neurons were triturred and grown in the presence of 1.5 μM double-stranded mutant octamer oligonucleotide or 3 μM single-stranded oligonucleotides (3' → 5' or 5' → 3' strands) in the absence of NGF, and cell survival was determined 14 hr later. Data represent mean ± SD of three experiments performed in triplicate and are expressed as percentage of cells surviving compared to control cells that did not receive oligonucleotides. * P < 0.05 compared to other oligonucleotides (B), to a mutant oligonucleotide (C), or to the same oligonucleotides in the presence of NGF (D).
gel-shift patterns between \textit{in vivo} and \textit{in vitro} experiments. (i) The different sources of proteins used in binding (nuclear vs. total). Whole cell extracts often give different gel-shift patterns compared to nuclear extracts because of proteins in the cytoplasm that can alter DNA–protein binding. Attempts to perform whole cell extracts with PC12 cells so binding profiles could be compared between whole cell extracts and nuclear protein were not informative because components in the cytoplasm of PC12 cells interfered with DNA–protein binding. (ii) Cellular and developmental complexity of sympathetic ganglia \textit{in vivo}. Cell death \textit{in vivo} is not an isolated event and occurs simultaneously with other developmental events such as glial proliferation and target innervation; therefore, changes in gel-shift patterns \textit{in vivo} may reflect these other events or the response of cells in the ganglia (glia, fibroblasts, or macrophages) to neuronal death. (iii) The population of cells that serve as the predominant source of the DNA binding proteins (a predominantly dying population \textit{in vitro} and a predominantly living population \textit{in vivo}). About 33% of sympathetic neurons in \textit{vivo} die over a 3- to 4-day period, while \textit{in vitro} 90–95% of the PC12 cells die over a 1- to 2-day period with the majority dying during the first 24 hr. Therefore, \textit{in vitro} experiments reflect changes in a predominantly dying population of cells while \textit{in vivo} experiments reflect changes in a predominantly living population.

Data in the present study support the idea that altered protein binding to the octamer motif is an early event in neuronal cell death. A hypothetical scenario consistent with our data as well as with that of others (31, 37–39) for early events in neuronal cell death would be the following: (i) Removing NGF alters phosphorylation (or amount) of a number of proteins including an Oct-2-like transcription factor or protein associated with an Oct-2-like complex (see ref. 40). (ii) Changes in phosphorylation or a decrease in the level of Oct-2-like factor decreases DNA binding of Oct-2-like factor. (iii) One or more of the genes activated by loss of Oct-2-like repressor activity would initiate and/or participate in a cascade of molecular and biochemical events eventually resulting in cell death.

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