A Timetable of Events During Programmed Cell Death Induced by Trophic Factor Withdrawal from Neuronal PC12 Cells

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We describe a timetable of events during programmed cell death (PCD) in neuronal PC12 cells, specifically, Ras signaling, immediate-early gene (IEG) expression, DNA fragmentation and commitment to PCD. Commitment occurs over a period from 10–20 hr after NGF withdrawal. Ras signaling declines rapidly after NGF removal, reaching minimal levels within 2–4 hr, well before the onset of commitment. DNA fragmentation, detected by TUNEL reaction, begins about 24 hr after NGF withdrawal, well after all cells are committed, but coincident with the onset of cell dissolution previously determined by trypan blue exclusion (Mesner et al., 1992). Among the IEGs studied here, *c-jun* and *TIS21* are expressed within 6 hr after NGF withdrawal. Expression of *c-fos*, *egr-1*, and *TIS11* does not begin until 20 hr after NGF withdrawal. IEG expression generally ends by 24 hr after NGF withdrawal. The IEGs *TIS7* and *nur77* are not expressed during PCD, yielding a pattern distinct from that following other stimuli. An identical pattern of IEG expression occurs in non-neuronal PC12 cells deprived of serum, although expression begins at 10–14 hr after serum withdrawal. A similar IEG expression pattern was observed in Rat-1 fibroblasts, with various genes expressed 6–18 hr after serum withdrawal. In none of these cell types did expression of the stress-related gene *Hsp70* change following trophic factor withdrawal. The distinctive pattern of IEG expression described here should facilitate identification of intracellular regulatory signals active during PCD.

[Key words: apoptosis, programmed cell death, trophic factor, NGF, neurotrophic factor, immediate-early gene expression, PC12 cell, Ras]

Programmed cell death (PCD), or apoptosis, is a physiological process, typically marked by nuclear condensation and DNA fragmentation, used to eliminate supernumerary or deleterious cells. In the nervous system, survival depends upon availability of neurotrophic factors: neurons lacking sufficient trophic support undergo PCD (Oppenheim, 1991). NGF promotes the survival of various central and peripheral neurons *in vivo* and *in vitro* (Barde, 1989) and promotes survival and neuronal differ-

entiation of PC12 cells (Greene and Tischler, 1976; Greene, 1978).

Responses to NGF are mediated by the TrkA receptor tyrosine kinase (Kaplan et al., 1991a,b; Klein et al., 1991; Loeb et al., 1991), which activates several intracellular signaling molecules including Ras (Qiu and Green, 1991) and phosphatidylinositol-3 kinase (PI-3-K) (Carter and Downs, 1992; Ohmichi et al., 1992; Soltoff et al., 1992). Ras activation appears necessary for NGF promotion of PC12 neuronal differentiation (Hagag et al., 1986; Szeberényi et al., 1990). Expression of mutant activated Ras promotes neuronal differentiation (Bar-Sagi and Feramisco, 1985; Noda et al., 1985) and survival (Borasio et al., 1989; Rukenstein et al., 1991). PI-3-K activity is associated with promotion of survival by NGF (Yao and Cooper, 1995).

Unlike sympathetic neurons, PC12 cells can survive in serum-containing medium whether or not NGF is present. If maintained in serum-free medium in the presence of NGF, PC12 cells will undergo neuronal differentiation, and will die when NGF is withdrawn (Greene, 1978; Greene et al., 1986). The death of NGF-deprived neuronally differentiated PC12 cells resembles PCD in neurons—and in many non-neuronal cells—in that the death is blocked by inhibitors of RNA or protein synthesis (Tata, 1966; Lockshin, 1969; Pratt and Greene, 1976; Martin et al., 1988; Scott and Davies, 1990; Mesner et al., 1992). Non-neuronal PC12 cells die when serum is removed and, in contrast to the neuronally differentiated cells, their death is not blocked by RNA and protein synthesis inhibitors (Mesner et al., 1992). This and other similarities between neuronal PC12 cells and sympathetic neurons establish PC12 cells as a model for neuronal PCD.

The ability of protein synthesis inhibitors to prevent PCD suggests that gene expression is required for PCD. Indeed, activation of specific genes has been associated with PCD in several systems (Osbourne and Schwartz, 1994), for example, *c-fos*, *c-myc*, and TRPM-2 are expressed in prostate epithelium during castration-induced regression (Buttayan et al., 1988) and cyclin D1 is expressed in NGF-deprived sympathetic neurons (Freeman et al., 1994). Immediate-early gene (IEG) expression is associated with PCD: examples include *nur77* expression during T-cell hybridoma death induced by ligation of antigen receptors (Liu et al., 1993; Woronicz et al., 1993) and *c-fos* expression during PCD in many neuronal and non-neuronal tissues (Smeyne et al., 1993). In sympathetic neurons, expression of IEGs of the Fos and Jun families, as well as *c-myc* and *egr-1*, has been detected following NGF deprivation *in vitro* (Estus et al., 1994), expression of *c-jun* was detected following lesion *in vivo* (Koistinaho et al., 1993). Moreover, expression of *c-jun* appears necessary for sympathetic neuronal PCD (Estus et al., 1994; Ham et al., 1995).

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As an initial step in determining causal relationships among PCD-associated events such as DNA fragmentation and gene expression, it is necessary to define their temporal relationships to each other, to termination of trophic factor-dependent intracellular signaling, and to commitment to cell death. We investigated these issues using, primarily, neuronally differentiated PC12 cells. We describe a distinctive pattern of IEG expression following trophic factor withdrawal and place it within the temporal context of critical post-withdrawal events: commitment to cell death, cessation of Trk-induced intracellular signaling, and onset of cell dissolution.

Materials and Methods

Cell culture and trophic factor deprivation. PC12 cells (passages 29–34) were maintained as described in (Greene and Tischler, 1976) on collagen-coated tissue culture dishes in RPMI 1640 medium containing 10% fetal bovine serum (GIBCO), 5% donor horse serum (JRH Biosciences), 100 U/ml penicillin and 100 µg/ml streptomycin; medium changed three times weekly. Sera were heat-inactivated at 56°C for 30 min. NGF was prepared from male mouse submaxillary glands, essentially as described by Mobley et al. (Mobley et al., 1972). Neuronal PC12 cell cultures were prepared by treating PC12 cells with 50 ng/ml NGF for at least 2 weeks (Greene et al., 1986). In these experiments, NGF treatment was initiated by replating PC12 cells, at 2.5×10^6 cells/100 mm dish, in RPMI 1640 medium containing 1% heat-inactivated donor horse serum and NGF. This small amount of serum provides no long-term trophic support but is used to facilitate changing the medium. The serum eliminates surface tension that can sometimes cause cells to peel off the plate when serum-free medium is repeatedly exchanged.

The total time allowed for neuronal differentiation in NGF was generally 15 d. The cells were maintained for 12 d in RPMI containing 1% horse serum and NGF (changed three times per week). The culture medium was then changed to 50 ng/ml NGF in serum-free RPMI. The cells were cultured in this medium without serum for an additional three days to facilitate subsequent complete withdrawal of trophic factor. NGF deprivation was accomplished by washing the cells twice with sterile phosphate-buffered saline (PBS) and then maintaining the cells in RPMI until the cells were counted.

Non-neuronal PC12 cells at moderate density ($1\text{--}2 \times 10^7$ cells/100 mm dish) were deprived of serum by washing twice with sterile PBS and then maintaining the cells in serum-free RPMI until the cells were counted. The cells were at equal density in each experiment.

Rat-1 fibroblasts (passages 18–24) were maintained in DMEM containing 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin, on untreated plastic tissue culture dishes. For trophic factor deprivation, subconfluent cells in 100 mm dishes were washed twice with sterile PBS and then maintained in serum-free DMEM until the cells were counted. The cells were at equal density in each experiment.

Cell death assay. To count viable cells, cells were removed from dishes by trituration in their culture medium (to avoid loss of detached cells). Cells were concentrated by brief centrifugation ($1000 \times g$, 5 min), the supernatant was removed, and the cells resuspended in 100 µl of PBS and 100 µl of trypan blue solution (0.4% trypan blue, 0.81% NaCl and 0.06% K_2HPO_4). Live (trypan blue-excluding) cells were counted using a hemacytometer.

Determination of Ras activity. PC12 cells were plated in 35 mm wells (1.5×10^6 cells/well) 2–3 d before an experiment. The cells were not exposed to fresh serum after plating as this alone is sufficient to increase Ras-GTP levels. The wells were washed with HBSG (HEPES-buffered saline + glucose: 137 mM NaCl, 2.7 mM KCl, 1.2 mM $CaCl_2$, 0.5 mM $MgCl_2$, 1 mg/ml glucose, 25 mM HEPES, pH 7.4) and ^{32}P - PO_4 (ICN) was added in HBSG (200–300 mCi/ml). The cells were incubated in ^{32}P - PO_4 for 2 hr at 37°C. The incubations were stopped by rapid removal of medium and washing with ice-cold phosphate-buffered saline (PBS). The cells were lysed in 0.5 ml of ice-cold lysis buffer A (1% Triton X-100, 100 mM NaCl, 5 mM $MgCl_2$, 10 mM benzimidazole (Calbiochem), 1 mg/ml BSA, 10 mg/ml leupeptin, 10 mg/ml aprotinin, 50 mM HEPES, pH 7.4). The lysate was incubated for 3 hr on a rotator with protein A-sepharose (Pharmacia) (35 µl/sample) precoated with rabbit anti-rat IgG. The sepharose was removed by a 5 min centrifugation in a micro-centrifuge. Ras was immunoprecipitated with culture supernatant (45 µl/sample) from hybridoma Y13-259 (Furth et al., 1982). The Y13-259

supernatant or control culture supernatant was added to the lysate and incubated on ice for 60 min. Rabbit anti-rat protein A-sepharose (25 µl/tube) was then added and the mixture incubated overnight on a rotator at 4°C. The immunoprecipitate was washed eight times with 1 ml of wash buffer (500 mM NaCl, 5 mM $MgCl_2$, 0.1% Triton X-100, 0.005% SDS, 50 mM HEPES, pH 7.4). Guanine nucleotides were eluted from the immunoprecipitate with elution buffer (2 mM EDTA, 2 mM dithiothreitol, 0.2% SDS, 0.5% GTP, 0.5% GDP). The eluted guanine nucleotides were separated on PEI-cellulose thin-layer chromatography (TLC) plates run in 0.75 M KH_2PO_4 , pH 3.4 (Satoh, 1988 #266) and the positions of GTP and GDP were identified with a short-wave UV lamp. The plates were then dried and exposed to preflashed Kodak XAR-5 film with a Du Pont Lightning Plus intensifying screen at $-86^\circ C$. Autoradiograms were scanned on a Hoefer densitometer for quantitation of radioactivity. The molar ratio of GTP to GDP was corrected for the additional phosphate in GTP based on the assumption that all positions are equally labeled with ^{32}P .

RNA extraction and Northern blotting. Polyadenylated RNA was prepared from cultured cells using oligo(dT) cellulose spin columns (Badley et al., 1988). Samples of mRNA (2 µg) were applied to 1.1% denaturing agarose gels for electrophoresis and vacuum transferred to ZetaProbe GT Nylon membranes (Bio-Rad). Blots were hybridized with NaOH-denatured, ^{32}P -labeled cDNA probes and exposed to Kodak X-Omat AR x-ray film (Kodak) with two Du Pont Lightning Plus intensifying screens (Dupont) for 12–48 hr at $-86^\circ C$. Probes were prepared by random hexamer priming of rat *egr-1* (NGFI-A) (Milbrandt, 1987), rat *nur77* (NGFI-B) (Milbrandt, 1988), human *c-myc* (Dalla-Favera et al., 1982), murine *hsp70* (S. Shtang), murine *c-jun* (Lampf et al., 1988), rat *c-fos* (Curran et al., 1987), rat GAPDH (Tso et al., 1985), murine *TIS7* (Varnum et al., 1989), murine *TIS21* (Fletcher et al., 1991), and rat *TIS11* (Kaneda et al., 1992).

Western blotting. Protein samples (20 µg) were separated by SDS-PAGE and transferred to Trans Blot nitrocellulose (Bio-Rad). After transfer, the filters were briefly stained with 0.05% w/v Ponceau red S in 3% TCA, and the position of the molecular weight standards was marked. The filters were then blocked for at least 2 hr in 1% bovine serum albumin (BSA) in TBST (Tris-buffered saline with 0.05% Tween 20). Primary antibody, anti-*egr-1* (Santa Cruz Biotechnology) diluted 1:100 in 1% BSA in TBST was incubated with the filter, in a humidified container, for 2 hr at 37°C. The blot was then washed once briefly in TBST and 3×15 min in TBST containing 1% casein. The filter was then incubated with alkaline phosphatase conjugated anti-rabbit IgG (1:2000, Sigma), in a humidified container for 3 hr at 37°C. The filter was then washed as above, followed by a 15 min wash in TBST containing 0.1 mM $MgCl_2$ and 0.4 mM $ZnCl_2$. Blots were developed in alkaline phosphatase buffer (100 mM Tris, 100 mM NaCl, 5 mM $MgCl_2$) containing 230 µg/ml BCIP and 165 µg/ml nitro blue tetrazolium.

TUNEL (terminal dUTP-biotin nick end labeling) assay. A protocol modified from that of Gavrieli et al. (Gavrieli et al., 1992) was used. Neuronal PC12 cells were grown at low density in collagen-coated 96-well plastic tissue culture dishes. Following complete neuronal differentiation (2 weeks), NGF was withdrawn by rinsing the cells four times with RPMI 1640. The cells were then maintained for various times in serum-free RPMI 1640 containing anti-NGF antiserum (1:2000 dilution, Sigma).

Cells were fixed in 10% formalin, 5% sucrose overnight and then permeabilized with 0.1% Triton X-100 in PBS for 10 min. Cells were rinsed for 10 min in distilled water followed by two 10 min washes in terminal transferase buffer (30 mM Tris pH 7.2, 1 mM $CoCl_2$, 140 mM sodium cacodylate). The terminal transferase labeling reaction was performed in terminal transferase buffer containing 20 µM dUTP + 4 µM biotinylated dUTP (Boehringer Mannheim) and 100 U/ml of terminal transferase (Boehringer Mannheim) at 37°C for 1 hr. The reaction was terminated by rinsing for 15 min in 300 mM NaCl/30 mM sodium citrate, then 10 min in PBS + 10 mg/ml BSA. Terminal transferase labeling was visualized by incubation with 1.25 µg/ml HRP-Z-avidin (Zymed) + 1 mg/ml BSA in PBS for 30 min at 37°C; then washing 2×10 min with PBS + 1 mg/ml BSA, followed by a 10 min wash with 100 mM sodium acetate buffer (pH 5.2). The cells were then incubated with 1.25 mM 3-amino-9-ethylcarbazole (AEC) + 0.03% H_2O_2 in 100 mM sodium acetate buffer for 5–10 min. at room temperature. Progress of the AEC reaction was monitored with a microscope and terminated by replacing the reaction solution with PBS. Labeled and unlabeled cells were counted with a Nikon Diaphot inverted microscope.

Figure 1. Commitment to PCD of neuronal PC12 cells deprived of trophic support for various lengths of time. Neuronal PC12 cell cultures were deprived of NGF as described in Materials and Methods. At various times after deprivation, NGF was readded to a concentration of 50 ng/ml. The cells were then maintained until 84 hr after the initial NGF withdrawal, at which time the number of viable cells was determined. Cell viability assays were performed by counting cells that exclude trypan blue. The data shown are from five separate experiments. Each experiment is represented by a different symbol. Error bars are SD of triplicate or quadruplicate samples. The solid line is a best fit third order polynomial. Since no commitment was observed at nine hours after withdrawal, commitment was not assayed prior to this time, as indicated by the broken line.

Results

Neuronal PC12 cell commitment to PCD following trophic factor deprivation

Commitment to cell death is defined as the time after which readdition of trophic support will no longer prevent cell death. Cell viability was assessed 84 hr after NGF withdrawal, at which time >80% of the cells have died. As shown in Figure 1, readdition of NGF up to 10 hr after the initial withdrawal rescues all of the cells, that is, as many cells are viable after 84 hr as in control cultures from which NGF was not withdrawn. Increasing the interval between NGF withdrawal and readdition results in a decreasing number of viable cells 84 hr after withdrawal. Thus, neuronal PC12 cells become gradually committed to cell death over a period extending from 10 to 20 hr after NGF withdrawal. About 50% of the cells are committed to cell death at about 16 hr after withdrawal.

Ras activity declines rapidly in neuronal PC12 cells following trophic factor deprivation

The GTP-binding protein Ras plays a critically important role in linking the NGF receptor tyrosine kinase, TrkA, to cellular responses. Ras activation has been shown to be necessary for PC12 cell responses to NGF. Constitutively active Ras is sufficient to maintain survival of PC12 cells (Rukenstein et al., 1991) or sympathetic neurons (Borasio et al., 1989) even in the absence of NGF. Thus, Ras activity was chosen as the indicator of neurotrophic factor intracellular signaling.

Ras activity is determined as the ratio of Ras-GTP to Ras-GDP, as previously described (Qiu and Green, 1991), (Ras is active when bound to GTP, inactive when bound to GDP). Ras activity peaks within 5 min of initial NGF treatment of PC12 cells and then declines, but persists at a level significantly above prestimulus levels for at least 2 weeks of NGF treatment (M.-S. Qiu and S. H. Green, unpublished observations). Following NGF withdrawal from neuronally differentiated PC12 cells (described in Materials and Methods), Ras activity declines (Fig. 2). The decline continues for about 4 hr by which time Ras activity reaches a new steady-state level of about 35% of the steady-state level that had been maintained during long-term

NGF treatment. This new level is comparable to the level of Ras activity in unstimulated non-neuronal PC12 cells prior to NGF treatment and is the minimal level of Ras activity observed in PC12 cells. These data show that Ras activity is rapidly down-regulated in neuronal PC12 cells following NGF withdrawal.

A small increase in Ras activity is apparent shortly after NGF withdrawal (Fig. 2). This was observed in all three replicate experiments performed but its significance is not clear. While it may indicate signaling due to NGF withdrawal, this small effect may simply be an artefact of the change of medium.

DNA fragmentation begins at 24 hr after NGF withdrawal from neuronal PC12 cells

Our previous studies (Mesner et al., 1992), which used trypan blue staining, suggested that the loss of viability begins at about 24 hr after NGF withdrawal from neuronal PC12 cells. DNA fragmentation is a phenomenon that accompanies cell death in many diverse cell types (Wyllie et al., 1984; McConkey et al., 1989; Kure et al., 1991). Since it is likely to be an irreversible phenomenon, we wished to determine whether DNA fragmentation coincides with commitment or with loss of cell viability. If the former, DNA fragmentation could be causal to commitment to PCD, if the latter, DNA fragmentation is more likely to be part of the process of cell dissolution. Our previous electrophoretic analysis suggested that DNA cleavage was not detectable as a nucleosomal ladder in NGF-deprived neuronal PC12 cells or SCG neurons (Mesner et al., 1992). However it was possible that DNA fragmentation resulting in larger fragments (e.g., >20 kbp) was occurring and was not detected by our assay methods. We have therefore reexamined DNA cleavage with the TUNEL technique of Gavrieli et al. (Gavrieli et al., 1992), which specifically labels the DNA ends generated by endonuclease activity. This technique has an advantage over electrophoretic analysis in that DNA fragmentation can be detected even if the DNA is being cleaved into pieces too large to be resolved on conventional agarose gels.

In control NGF-treated neuronal PC12 cell cultures a background level of about 10% TUNEL-positive cells (detected as red-stained nuclei) was observed (Fig. 3). The fraction of TU-

Figure 2. Ras activity in neuronal PC12 cells following NGF withdrawal. Ras was immunoprecipitated from lysates of ^{32}P -labeled neuronal PC12 cell cultures at various times after NGF withdrawal. Bound guanine nucleotide was quantified as described in Methods. The basal GTP:GDP ratio associated with control cells from which NGF was not withdrawn is assigned a value of 1.0. The data shown are from a single experiment, typical of three performed.

NEL-positive cells remained at this background level for about 24 hr after NGF withdrawal. After 24 hr, the proportion of TUNEL stained cells increased dramatically (Fig. 3) indicating an occurrence of DNA fragmentation in these NGF-deprived neuronal PC12 cells. After 2 d without trophic support, by which time degenerative morphological changes were easily detectable with phase microscopy, about 40% of cells were TUNEL positive. In cultures deprived of NGF for 3 d, when cell viability measured by trypan blue exclusion is less than 36% of control (Mesner et al., 1992), approximately 70% of cells were TUNEL positive (Fig. 3). We find that neuronal PC12 cells are entirely committed to the cell death fate by about 20 hr after NGF withdrawal. Sympathetic neurons are similarly committed by 18–24 hr after NGF withdrawal (Martin et al., 1992; Deckwerth and Johnson, 1993; Edwards and Tolkovsky, 1994). Since the number of TUNEL positive cells does not increase until 24 hr after NGF withdrawal it appears that significant amounts of DNA degradation begin only after the time of commitment to PCD. Our present observations would therefore support the conclusion

that DNA fragmentation occurs as a result of commitment to PCD rather than as a cause.

Differential immediate early gene expression in NGF-deprived neuronal PC12 cells

We have previously observed that PCD in neuronal PC12 cells is, at least in part, transcription-dependent (Mesner et al., 1992). Induction of IEGs typically accompanies alterations in a cell's pattern of gene expression. Expression of various IEGs has been previously noted to accompany the process of cell death (Buttayan et al., 1988; Buttayan et al., 1989; Liu et al., 1993; Woronicz et al., 1993). We therefore assayed the expression of various IEGs in neuronal PC12 cells to determine when transcriptional events occur following NGF withdrawal.

Northern blot analysis of neuronal PC12 cell cultures deprived of NGF for various times revealed the induction of several IEGs (Fig. 4). Expression of the genes *egr-1* (NGFI-A/Zif268), *c-fos*, *TIS11*, and *TIS21*, barely detectable in control cells or in cells deprived of NGF for up to 18 hr, exhibits a dramatic four- to

Figure 3. DNA fragmentation in neuronal PC12 cells following NGF withdrawal. Cells containing fragmented DNA were visualized by TUNEL labeling (Gavrieli et al., 1992). The proportion of TUNEL-positive cells was determined by counting cells with a microscope. Error bars represent SD of quadruplicate data.

Figure 4. IEG expression in neuronal PC12 cells following NGF withdrawal. Polyadenylated mRNA (2 μ g/lane) was prepared from neuronal PC12 cell cultures deprived of NGF in serum-free medium for various times. Blots were probed for expression of IEGs indicated. The Hsp70 probe was employed to assess expression of stress-related genes and GAPDH to assess mRNA integrity and to compare loading among the lanes. mRNA from PC12 cells treated with NGF at 50 ng/ml for 1.5 hr was employed as a positive control for IEG expression.

Figure 5. IEG expression in non-neuronal PC12 cells following serum withdrawal. Polyadenylated mRNA (2 μ g/lane) was prepared from non-neuronal PC12 cell cultures deprived of serum for various times. Blots were probed for expression of the IEGs indicated. The Hsp70 probe was employed to assess expression of stress-related genes and GAPDH to assess mRNA integrity and to compare loading among the lanes. mRNA from PC12 cells treated with NGF at 50 ng/ml for 1.5 hr was employed as a positive control for IEG expression.

fivefold induction approximately 22 hr after NGF-withdrawal. The observed inductions appear to be transient; transcript levels were significantly lower by 26 hr after NGF withdrawal. After 48 hr of NGF deprivation expression levels of *egr-1*, *TIS11*, and *TIS21* had returned to the basal level of prewithdrawal expression, whereas *c-fos* expression remained two to three times the basal level. The expression of *c-jun*, *c-fos* and *TIS21* followed a more complex pattern than that of *egr-1* and *TIS11*. Although a peak of *c-jun*, *c-fos* and *TIS21* expression also occurred at 22 hr after withdrawal, significant, although variable expression, was observed in some experiments as early as 6 hr after NGF deprivation. Following the peak of expression at 22 hr after withdrawal, expression declined to basal levels as did *egr-1* and *TIS11* earlier, throughout the commitment period (Fig. 4).

The expression of IEGs at 6–22 hr postwithdrawal is a consequence of selective induction relative to GAPDH, not of selective stabilization of IEG transcripts. The yield of mRNA declines only slightly over this period, from $\approx 13 \mu$ g polyadenylated mRNA from 4×10^7 control cells to $\approx 10 \mu$ g polyadenylated mRNA obtained from 4×10^7 cells plated and deprived of NGF for 14 hr. After 24 hr in the absence of NGF, the yield of mRNA does decline: $\approx 7 \mu$ g of polyadenylated mRNA was obtained from 4×10^7 cells plated and deprived of NGF for 26 hr. However, IEG mRNA levels decline relative to GAPDH mRNA at this time, indicating that IEG transcripts are selectively degraded even as the amount of mRNA in the culture declines. By 48 hr after NGF withdrawal, $<4 \mu$ g mRNA could be obtained from 4×10^7 cells originally plated.

c-fos, *TIS11*, *egr-1*, *c-jun*, and *TIS21* are members of a set of genes expressed in PC12 cells in response to a variety of stimuli including EGF, phorbol esters, NGF and elevated intracellular calcium (Greenberg et al., 1985; Kujubu et al., 1987; Milbrandt, 1987), P. W. Mesner and S. H. Green, unpublished observations).

c-myc, *TIS7* (PC-4) and *nur77* (NGFI-B) are also induced by these stimuli (Greenberg et al., 1985; Milbrandt, 1988; Tirone and Shooter, 1989; P. W. Mesner and S. H. Green, unpublished observations). The expression pattern observed here following NGF withdrawal is distinctive in that expression of the latter genes does not occur (Fig. 4).

To address the possibility that the observed pattern of IEG induction was a consequence of PCD-associated cell stress or regulatory dysfunction, we analyzed the expression of the genes Hsp70 and GAPDH. No change in the level of expression of the stress-related gene Hsp70 was observed at any time after NGF withdrawal. Likewise, expression of the metabolic housekeeping gene GAPDH did not change after NGF withdrawal.

IEG expression in serum-deprived non-neuronal (chromaffin-like) PC12 cells

PCD in trophic factor-deprived non-neuronal PC12 cells is not inhibited by inhibitors of macromolecular synthesis. We nevertheless evaluated IEG expression in non-neuronal cells to compare it with that observed during neuronal cell PCD. Northern blot analysis was performed on non-neuronal PC12 cell cultures deprived of serum and maintained in serum-free medium for various times. Surprisingly, a pattern of IEG expression similar to that in NGF-deprived neuronal PC12 cells was observed: *egr-1*, *TIS11*, *TIS21*, *c-fos*, and *c-jun* were all transiently induced in serum-deprived non-neuronal PC12 cells (Fig. 5). The time of expression, following serum withdrawal, was accelerated somewhat relative to neuronal PC12 cells. A transient three- to four-fold increase in the expression of *egr-1*, *TIS11*, and *TIS21* mRNA was observed about 14 hr after serum-deprivation and a transient induction of *c-fos* was observed at about 18 hr after serum withdrawal. *c-jun* induction, was first detectable 18 hr

after serum withdrawal, and showed fluctuating and variable expression at all later time points. With the exception of *c-jun*, expression all of the IEGs that were induced in serum-deprived non-neuronal PC12 cells declined to undetectable levels after 22–26 hr.

As is the case for neuronal PC12 cells, the expression of IEGs in non-neuronal PC12 cells at 6–22 hr postwithdrawal is a consequence of selective induction relative to GAPDH, not of selective stabilization of IEG transcripts. The yield of polyadenylated mRNA ($\approx 4.4 \mu\text{g}/100 \text{ mm dish}$) was the same for control cells as for cells deprived of serum for 14 hr, the time at which maximal IEG expression was observed. The yield of mRNA dropped precipitously after 24 hr in serum-free medium, to $\approx 0.8 \mu\text{g}$ polyadenylated mRNA/100 mm dish at 26 hr and to $0.4 \mu\text{g}/100 \text{ mm dish}$ at 48 hr. IEG mRNA levels decline relative to GAPDH mRNA at this time, as is the case for neuronal PC12 cells, again indicating that IEG transcripts are selectively degraded even as the amount of mRNA in the culture declines.

The patterns of IEG induction in trophic factor-deprived neuronal and non-neuronal PC12 cells resemble each other in the lack of induction of *c-myc*, *TIS7* and *nur77* and the constitutive, unchanging expression of Hsp70 and GAPDH (Fig. 5). Despite the fact that non-neuronal PC12 cells die in a transcription and translation-independent manner, the overall pattern of IEG inductions is quite similar to that seen in NGF-deprived neuronal PC12 cells.

The major difference between neuronal and non-neuronal PC12 cells lies in the timing of IEG induction following withdrawal of trophic support. In neuronal PC12 cells the peak of IEG expression was at 20–22 hr after NGF withdrawal. In non-neuronal cells the peak occurred 14–18 hr after serum withdrawal (compare Figs. 4 and 5) 4–8 hr earlier than in neuronal PC12 cells. In a few experiments elevated expression of *egr-1*, *TIS11*, *TIS21*, *c-fos* and *c-jun* was detected as soon as 6 hr after serum withdrawal in non-neuronal PC12 cells (data not shown).

The presence of IEG expression in apoptotic non-neuronal PC12 cells is remarkable because it has been previously shown (Rukenstein et al., 1991; Mesner et al., 1992) that these cells die by an transcription-independent mechanism. Specifically, our previous results (Mesner et al., 1992) showed that the death of non-neuronal PC12 cells was not inhibited by treatment with cycloheximide (CHX) or actinomycin D (AMD) at concentrations that reduced ^{35}S -methionine incorporation into TCA-precipitable material to about 2% of control levels (Martin et al., 1988; Mesner et al., 1992). However, under such conditions IEG transcription is induced because the negative feedback effect of IEG protein products on transcription of the genes encoding them is removed, a phenomenon known as “superinduction” (Greenberg et al., 1986). Superinduction might allow sufficient protein synthesis of IEG products to effect cell death in non-neuronal PC12 cells, negating the effect of the macromolecular synthesis inhibitors. We assayed the level of *egr-1* protein by Western blotting in non-neuronal PC12 cells treated with NGF in the presence or absence of CHX or AMD. This treatment was chosen rather than NGF withdrawal because IEG expression is at a greater level following trophic factor addition than it is during PCD, making it more likely that some expression might escape inhibition by CHX or AMD.

Cotreatment of non-neuronal PC12 cells with NGF and CHX or AMD (both inhibitors at $3 \mu\text{g}/\text{ml}$) reduced *egr-1* expression below the level of detection, relative to the NGF-treated control (Fig. 6). Our results indicate that the concentrations of CHX and

Figure 6. Expression of *egr-1* protein in non-neuronal PC12 cells is blocked by inhibitors of macromolecular synthesis. Chromaffin-like PC12 cell cultures untreated (0), or treated for 1.5 hr with 50 ng/ml NGF alone (N), NGF + $3 \mu\text{g}/\text{ml}$ cycloheximide (NGF/CHX), or NGF + $3 \mu\text{g}/\text{ml}$ actinomycin D (NGF/ActD). The cells were lysed in SDS-PAGE sample buffer; 20 μg were separated by SDS-PAGE and transferred to nitrocellulose. Western blotting was performed as described in Materials and Methods.

AMD previously used to block protein synthesis in trophic factor-deprived non-neuronal cells did effectively block IEG expression. Thus, it is likely that non-neuronal PC12 cells can undergo PCD without gene expression even though an IEG expression pattern similar to that in neuronal PC12 cells was observed here.

IEG expression in serum-deprived Rat-1 fibroblasts

The above observations suggest that the pattern of IEG expression during PCD is very similar to that in neuronal and non-neuronal cells. To further explore this possibility, we analyzed IEG expression in trophic factor-deprived Rat-1 fibroblasts. These cells have been previously used for studies of apoptosis (e.g., Evan et al., 1992) because they undergo PCD following serum withdrawal. Northern blot analysis was performed on cultures deprived of serum and maintained in serum-free medium for various lengths of time. In general, the pattern of IEG expression in serum-deprived Rat-1 fibroblasts, with respect to the identity of genes induced, is similar to that observed in non-neuronal and neuronal PC12 cell cultures following trophic factor deprivation. The only exception is a variable expression of *nur77* in Rat-1 cells. Although the patterns of IEG expression in Rat-1 cells were much more variable than in PC12 cells, we consistently found elevated levels of *egr-1*, *TIS11*, *TIS21*, *c-fos*, and *c-jun* mRNAs after serum withdrawal (Fig. 7). Expression of *egr-1* increased four- to fivefold between 10–18 hr of serum deprivation, after which expression declined to $2\text{--}3 \times$ control by 22 hr after withdrawal and remained at this level. Levels of *TIS11*, *TIS21* and *c-fos* mRNAs were increased approximately two- to threefold after 6–10 hr of trophic factor deprivation. Expression of these three IEGs remained constant until about 22–26 hr after serum withdrawal, then declined to basal levels. As in PC12 cells *c-jun* expression was quite variable but a two- to threefold induction could be observed about 14–18 hr after serum-withdrawal. *c-jun* expression declined to basal levels by 26 hr after serum withdrawal.

The yield of mRNA declines slowly after serum withdrawal from Rat-1 cells. The yield from control cells, $\approx 0.5 \mu\text{g}/100 \text{ mm dish}$, is approximately the same as the yield from cells deprived of serum for 14 hr, $\approx 0.44 \mu\text{g}/100 \text{ mm dish}$, indicating that the increase in IEG expression at this time is the result of selective induction relative to GAPDH mRNA. The yield of mRNA declines to $\approx 0.3 \mu\text{g}/\text{dish}$ at 26 hr and to $\approx 0.25 \mu\text{g}/\text{dish}$ at 48 hr.

As was the case in PC12 cells, induction of *TIS7* and *c-myc* did not generally occur after serum withdrawal from Rat-1 cells,

Figure 7. IEG expression in Rat-1 fibroblasts following serum withdrawal. Polyadenylated mRNA (2 μ g/lane) was prepared from Rat-1 fibroblast cultures deprived of serum for various times. Blots were probed for expression of IEGs indicated. The Hsp70 probe was employed to assess expression of stress-related genes and GAPDH to assess mRNA integrity and to compare loading among the lanes. Positive control mRNA was prepared from Rat-1 fibroblasts treated with 100 μ g/ml TPA for 2.0 hr.

although, in some cases, Rat-1 cultures did exhibit a two- to threefold increase in the level of *c-myc* mRNA after 48 hr in serum-free medium. Unlike PC12 cells, Rat-1 cells may express *nur77* following trophic factor withdrawal. *nur77* expression could be detected as early as 6 hr after serum withdrawal. However expression of *nur77* was variable and not observed in some Rat-1 cultures. As in PC12 cells, no change in Hsp70 or GAPDH expression was detected in trophic factor-deprived Rat-1 cells, indicating that the IEG expression is not a stress response nor a generalized transcriptional activation.

Discussion

This study describes a timetable of molecular events occurring in neuronal PC12 cells following trophic factor deprivation. We have identified a pattern of IEG expression characteristic of neuronal PCD and placed it within the temporal context of events following withdrawal of NGF from neuronal PC12 cells (Fig.

8). These events include cessation of intracellular trophic signaling, commitment to the cell death fate, and the actual death of the cells—as determined here by appearance of DNA fragmentation and previously (Mesner et al., 1992) by loss of trypan blue exclusion. We find that neuronal PC12 cells become committed to PCD over a period 10–20 hr following NGF withdrawal. This is similar to the commitment time course previously reported by Pittman and colleagues (Pittman et al., 1993) for a variant PC12 cell line. The commitment time courses for neuronal PC12 cells closely resemble those reported for rat sympathetic neurons (Martin et al., 1992; Deckwerth and Johnson, 1993; Edwards and Tolkovsky, 1994), further establishing neuronal PC12 cells as a reliable model for neuronal PCD.

Ras activity declines rapidly after withdrawal of trophic factor, within 2–4 hr, well before the onset of commitment to PCD. While *c-jun* and *TIS21* are initially expressed prior to commitment, inductions of some IEGs, for example, *c-fos*, *TIS11*, and *egr-1* do not occur until the end of the commitment period. DNA fragmentation and cell dissolution begin about 24 hr after NGF withdrawal, at least 4 hr after all cells are committed to PCD.

Trophic signaling and commitment to PCD

Our data show a gap of at least 6 hr between the cessation of Ras activity and the earliest time at which cells begin to become committed to PCD. An important question is what process(es), evidently reversible, occupy this period. One possibility is that other protein-tyrosine kinase effectors are more persistent than Ras after NGF is removed. This is an important consideration given that PI-3-K has been implicated as a primary effector of trophic signaling (Yao and Cooper, 1995). However, tyrosine phosphorylation of high molecular weight proteins declines at least as fast as does Ras activity after transient activation by EGF (Qiu and Green, 1992), making it unlikely that protein-tyrosine kinases activate PI-3-K or other effectors longer than they do Ras.

Another possibility is that protein kinases downstream of Ras continue to signal for some time after cessation of Ras activity and that it is their eventual inactivation that establishes commitment. This seems unlikely given that kinases of this sort, MAP kinases (Qiu and Green, 1992; Heasley, 1992, #5642; Nguyen, 1993, #6291) and S6 kinases (Nguyen et al., 1993), inactivate with a time course similar to Ras inactivation following transient activation by EGF. Furthermore, although NGF induced MAP kinase activity persists for several hours (Qiu and Green, 1992; Heasley, 1992, #5642; Nguyen, 1993, #6291), it

Figure 8. Diagrammatic representation of the timing of events following NGF withdrawal from neuronally differentiated PC12 cells. Ras activity declines rapidly after NGF withdrawal. Cells gradually become committed to cell death over a period of time from 10–20 hr after NGF withdrawal. IEG expression occurs at various times after NGF withdrawal but expression of some genes occurs only at the end of—or after the commitment period. Actual loss of cells does not begin until at least 24 hr after NGF withdrawal and continues for several days thereafter.

returns to near prestimulus levels after several days of NGF treatment (Tsao and Greene, 1991; M. S. Qiu and S. H. Green, unpublished observations). Thus, MAP kinase activity is already at a minimal level in neuronally differentiated PC12 cells before NGF withdrawal.

Alternatively, the interval between cessation of Ras signaling and onset of commitment to PCD may be due to the gradual degradation or inactivation of a constitutively active molecule that prevents PCD. Consistent with such a mechanism, proteases have been implicated in PCD in several diverse systems, including *C. elegans* (Yuan, 1993, #6579), insect larval muscle (Schwartz et al., 1991), mammalian fibroblasts (Miura et al., 1993), and neurons (Gagliardini et al., 1994), and cell killing by T cells (Heusel et al., 1994).

Buttayan et al. (Buttayan et al., 1988) have identified "cascades" of transcriptional inductions, involving IEGs and other genes, in PCD in prostate gland. Possibly, such transcriptional cascades start soon after trophic signaling ceases. If so, the early transcriptional events must not be sufficient for cell death because they are occurring long before the commitment.

DNA fragmentation

Endonuclease activity resulting in DNA fragmentation is typically a feature of PCD (Wyllie, 1980). Edwards et al. (Edwards and Tolkovsky, 1994) reported that commitment to PCD was concomitant with increased nucleolytic activity, implying that DNA fragmentation was the proximate cause of commitment. The present study argues against a causal role for DNA fragmentation in commitment to PCD. By using TUNEL staining as a direct and sensitive assay of DNA fragmentation, we find that the increase in TUNEL reactivity begins at ≈ 24 hr after NGF withdrawal, approximately 4 hr after all cells have become committed to cell death. Our previous studies (Mesner et al., 1992) indicate that this is the same time at which loss of ability to exclude trypan blue is first detected. These observations imply that DNA fragmentation is associated not with commitment but, rather, with dissolution of the cell.

In previous studies of our neuronal PC12 cells, we were unable to detect an oligonucleosomal DNA "ladder" at any time after NGF withdrawal, including ≥ 24 hr postwithdrawal when TUNEL reactivity was detected here. The presence of TUNEL reactivity without a detectable DNA ladder suggests that DNA is being cleaved into large fragments not resolved by standard agarose gels (e.g., ≥ 50 kbp fragments). Even such a limited level of fragmentation would yield more than 10^4 DNA termini/nucleus capable of being labeled by TUNEL. Batistatou and Greene (Batistatou and Greene, 1991) have observed an oligonucleosomal ladder in PC12 cells undergoing PCD. Perhaps DNA fragmentation is occurring in all cases, but PC12 cells maintained in different laboratories differ in ability to degrade DNA to smaller, oligonucleosomal fragments. Similarly, a murine fibroblast clone has been described (Ucker et al., 1992) that does not exhibit a DNA ladder during T cell-induced PCD. In that case differences among the clonal lines appears to be due to differential expression of DNase I-like endonuclease activities.

IEGs expressed during PCD

Although our observation of *TIS11* and *TIS21* induction during neuronal PCD is novel, several studies have documented induction of *egr-1*, *c-fos*, and *c-jun*, and other IEGs, in dying neuronal cells. Expression of *c-fos* accompanies PCD in many cell types *in vivo* and *in vitro* (Smeyne et al., 1993). Estus et al. (Estus et

al., 1994) reported induction of NGFI-A (*egr-1*), *c-jun*, *c-fos*, *fos B*, *jun B*, and *c-myc* in NGF-deprived, cultured rat sympathetic neurons. Similarly, Koistinaho et al. (Koistinaho et al., 1993) detected *c-jun* expression in lesioned sympathetic neurons *in vivo*.

While IEGs are expressed in response to many different stimuli, the pattern of expression differs among stimuli or among cells (Herschman, 1989). Thus, expression of a particular IEG is unlikely to be diagnostic of a particular cellular response, but expression of a particular pattern of IEG expression can be. The pattern observed here in neurotrophic factor-deprived PC12 cells is like that in sympathetic neurons (Estus et al., 1994), implying that this is a diagnostic pattern for neuronal PCD. *egr-1*, *c-fos*, *c-jun*, *TIS11*, *TIS21*, *TIS7*, *c-myc*, and *nur77* are coexpressed in PC12 cells in response to a wide variety of stimuli including the mitogen EGF, the neurotrophic factor NGF, and increased intracellular calcium (Greenberg et al., 1985; Kujubu et al., 1987; Milbrandt, 1987, 1988; Bartel et al., 1989; Mesner and Green, unpublished observations; Altin et al., 1991). In contrast, *c-myc* and *nur77* are not expressed with the other genes during PCD in neuronal or non-neuronal trophic factor-deprived PC12 cells. Similarly, *c-myc* and *nur77* are not expressed during PCD in sympathetic neurons (Estus et al., 1994; Freeman et al., 1994).

Lack of *nur77* expression is also seen in T-cell hybridomas undergoing PCD following IL-2 withdrawal (Liu et al., 1993; Woronicz et al., 1993). Interestingly, T-cell hybridomas induced to undergo PCD by ligation of antigen receptors express *nur77* and such expression appears to be required for PCD. This may indicate mechanistic differences between PCD induced by an extracellular signal and that induced by withdrawal of trophic factor. In addition, there may be differences among cells in the pattern of IEGs expressed during PCD. This is consistent with our observation of such differences, albeit small, between PC12 and Rat-1 cells.

The timing of IEG expression during PCD

While similar genes are induced in neuronal and non-neuronal PC12 cells, following trophic factor withdrawal, the temporal pattern is different. In neuronal PC12 cells, some IEGs—for example, *c-jun* and *TIS21*—are expressed within 6 hr after NGF withdrawal; others—for example, *egr-1*, *c-fos*, and *TIS11*—are not expressed until ≈ 20 hr after NGF withdrawal, at the end of the commitment period. Non-neuronal PC12 cells exhibit IEG inductions over a range of time from 6–14 hr after serum withdrawal. This pattern reflects the time course of loss of cell viability following trophic factor withdrawal from PC12 cells (Mesner et al., 1992); cell death appears to occur at a slower pace in neuronal PC12 cells than in non-neuronal PC12 cells. The death time course in neuronal PC12 cells resembles that in sympathetic neurons (Martin et al., 1992; Deckwerth and Johnson, 1993; Edwards and Tolkovsky, 1994).

Requirement for IEG expression during PCD

Previous studies have implied a requirement for IEG expression, particularly *c-jun*, in neuronal PCD (Estus et al., 1994). A striking difference between neuronal and non-neuronal PC12 cells is their dependence on macromolecular synthesis during PCD. Transcription and translation inhibitors that effectively abolish IEG expression block cell death in trophic factor deprived neuronal PC12 cells but not in non-neuronal PC12 cells. Thus, the presence of IEG expression during PCD does not imply a requirement for such expression in all cells.

The patterns of IEG expression described here presumably mirror intracellular signals and regulatory processes that are active at different times during the process of PCD. The different, complex expression patterns in the three cell types studied here imply complex regulation with different signals active in a sequence throughout the PCD process. Our description of these expression patterns thus provides a means to identify the intracellular signals active at different times during PCD. For example, the IEG expression pattern observed here argues against a role for elevated cytosolic Ca^{2+} in PCD. *nur77* is strongly induced by depolarization or Ca^{2+} ionophore in PC12 cells (Bartel et al., 1989; Hazel et al., 1991; Mesner and Green, unpublished observations) but is not induced during PCD.

Thus, while expression of these IEGs may not be necessary for cell death in all cell types, their identification nonetheless provides a valuable means to monitor and identify intracellular regulatory events that occur during PCD. Facilitating this endeavor is the fact that considerable information regarding the regulation of many IEGs already exists, including descriptions of flanking sequences containing their transcription regulatory elements.

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