Thyroid Hormone Receptor/c-erbA: Control of Commitment and Differentiation in the Neuronal/Chromaffin Progenitor Line PC12

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Abstract. The c-erbA proto-oncogenes encode nuclear receptors for thyroid hormone (T3), a hormone intimately involved in mammalian brain maturation. To study thyroid hormone receptor (TR) action on neuronal cells in vitro, we expressed the chicken c-erbA/TRα-1 as well as its oncogenic variant v-erbA in the adrenal medulla progenitor cell line PC12. In the absence of T3, exogenous TRα-1 inhibits NGF-induced neuronal differentiation and represses neuron-specific gene expression. In contrast, TRα-1 allows normal differentiation and neuronal gene expression to occur in the presence of T3. Finally, TRα-1-expressing cells become NGF-responsive for proliferation when T3 is absent, but NGF-dependent for survival in presence of T3. A similar differentiation induction by NGF plus T3 was observed in a central nervous system-derived neuronal cell line (E 18) expressing exogenous TRα-1. Together with the finding that TRα-1 constitutively blocked dexamethasone-induced differentiation of PC12 cells into the chromaffin pathway, these results suggest that TRα-1 plays an important role in regulating commitment and maturation of neuronal progenitors.

In contrast, the v-erbA oncogene, a mutated, oncogenic version of TRα-1, partially but constitutively inhibited NGF-induced neuronal differentiation of PC12 cells and potentiated dexamethasone-induced chromaffin differentiation, giving rise to an aberrant “interlineage” cell phenotype.
sensitive to thyroid hormones (Gould and Butcher, 1989). These cells also express maximum levels of the high affinity (gpl40s) NGF receptor (Holtzman et al., 1992) and thyroid hormone and NGF were found to cooperate in the development of specific cholinergic markers in basal ganglia (Patel et al., 1988) and other CNS areas (Clos and Legrand, 1990). These findings strongly suggest a key role of thyroid hormone receptors in neural tissue formation and maturation, probably involving cooperation with neurotrophins.

We have addressed this question by studying the effects of T3 on neuronal cells differentiating in culture. Unfortunately, appropriate neuronal cell lines from the CNS capable of in vitro differentiation in response to neurotrophic factors are missing and primary cells obtained from brain do not grow in culture for extended time periods, hampering long-term studies. We therefore employed the PC12 cell line that has been extensively used as a model system for neuronal differentiation since its establishment from a rat pheochromocytoma (Greene and Tischler, 1976). PC12 cells show characteristics of common precursor cells for sympathetic adrenergic neurons and neurosecretory epithelioid chromaffin cells. NGF induces PC12 cells to differentiate along the neuronal pathway, whereas glucocorticoid hormones promote their differentiation into chromaffin cells (Greene and Tischler, 1982; Levi et al., 1988). Thus, PC12 differentiation in vitro at least partially mirrors differentiation of respective neural crest-derived progenitor cells in the rat adrenal medulla in vivo (Doupe et al., 1985a,b; Anderson and Axel, 1986).

Because of their origin from a tumor, it was not surprising that PC12 cells behave aberrantly in several ways when compared to normal precursor cells in vivo. They continuously grow in the absence of neurotrophic factors, show an altered response to various exogenous stimuli and differentiate only partially into either neuronal or chromaffin cells. Also, they show no known response to thyroid hormone, a result also obtained in our own pilot experiments. This could be due to expression of low levels of c-erbA/Trα-1 together with high levels of c-erbA/Trα-2 RNA, which encodes a variant form of the receptor generated by differential splicing. Trα-2 is unable to bind hormone but efficiently blocks transcriptional activation by the active c-erbA/Trα-1 form (Koenig et al., 1989; Lazar et al., 1989; Rentoumis et al., 1990).

For these reasons, we decided to stably express an exogenous Trα-1 gene in PC12 cells using suitable retroviral vectors (Muñoz et al., 1990). The rationale behind this study was to restore Trα-1 expression in a neuronal progenitor cell line that express only low levels of Trα-1, most likely as a consequence of the immortalization process, and then analyze neuronal differentiation in response to thyroid hormone and neurotrophic factors. We show in this paper that exogenous Trα-1 expressed at physiological levels regulates NGF-dependent differentiation and gene expression of PC12 cells in a Trα-1 dependent fashion and constitutively blocks their entry into the chromaffin differentiation pathway. In addition, ligand-activated Trα-1 renders PC12 cells dependent on NGF as a neurotrophic factor. In contrast to Trα-1, v-erbA, a mutated, oncogenic version of Trα-1 that seems to act as a constitutive repressor of genes regulated by c-erbA/Trα-1 (Damm et al., 1989; Sap et al., 1989; Disela et al., 1991), constitutively inhibited both neuronal and chromaffin differentiation of PC12 cells.

Materials and Methods

Cells and Cell Cultures

A clonal subline of rat PC12 cells (referred to as 6c) was used throughout. This clone responded particularly well to differentiation induction by NGF in terms of neurite outgrowth on standard plastic dishes. (Whorton, C. W., and M. Busslinger, unpublished observations). PC12 6c cells were grown in DME supplemented with 10% horse serum, 5% FCS, and 1 mM glutamine (standard growth medium) (all from GIBCO, U.K.). Establishment and properties of the E 18 161 cell line derived by spontaneous immortalization from 17-d embryonic rat brain will be described elsewhere (Seliger, B., unpublished observations). Cells were grown in Ham's F12 medium supplemented with 10% FCS.

Retrovirus Vectors and Generation of erbA-overexpressing Cells

The recombinant retroviruses encoding the chicken c-erbA/TRα-1 and v-erbA genes used to infect PC12 6c cells have been already described (Muñoz et al., 1990). Both are derived from the Moloney murine leukemia virus and encode the neomycin-resistant gene providing resistance to the antibiotic G418. For infection, 5 × 10⁶ cells were plated in 60-mm dishes and incubated overnight with fibroblast-grown virus plus 8 μg/ml polybrene (Sigma Chem. Co., St. Louis, MO). Medium was then changed and selected with 800 μg/ml G418 (GIBCO BRL, Gaithersburg, MD) started 48 h after infection (Muñoz et al., 1990). Six clones of resistant TRα-1-infected cells could be expanded separately (cA1-cA6). cA4, the clone exhibiting the highest specific T3-specific binding was used in all experiments. In contrast, no individual v-erbA-expressing clones could be expanded. Therefore, a pool of G418 resistant, v-erbA-expressing clones was used.

Infection of E 18 161 cells with TRα-1 was done exactly as described for PC12 6c. Ten TRα-1-expressing clones were obtained, one of which showing reproducible, high TRα-1 expression as determined by T3 binding (E 18 cA9) was used in the experiments.

Induction of Neural and Chromaffin Differentiation

For induction of optimal neuronal differentiation, well suspended PC12 6c cells were seeded at 10⁴ cells per cm² in standard growth medium. Murine 15S NGF (Sigma Chem. Co.) was added at a concentration of 50 ng/ml every 2 d. Triiodothyronine (Sigma Chem. Co.) was added daily at a final concentration of 150 nM. Medium was changed partially or totally every 48 h. Chromaffin differentiation was induced by treatment with dexamethasone (5 μg/ml; Serva, Heidelberg) every 2 d or when the medium was changed.

Embryonic CNS neuroblasts (E 18 cA9) were induced to differentiate by seeding them in serum-free Dulbecco's plus Ham's F12 (1:1) medium supplemented with the additions of N2 differentiation medium (Bottenstein and Sato, 1979). NGF (50 ng/ml) was added every 2 d while T3 (150 nM) was added daily.

[3H]Thymidine Incorporation Assay

Cells were seeded into 35-mm dishes in 2 ml standard growth medium at a density of 8 × 10⁴ cells per dish and treated with NGF and/or T3 as described above. Cell monolayers were labeled for 3.5 h with 1 μCi [6-3H] thymidine (25 Ci/mmol; Amersham Intl., Buckinghamshire, U.K.), washed twice with PBS, precipitated with 10% trichloroacetic acid for 10 min, air-dried, and dissolved in 400 μl of 0.1% NaOH and 1% SDS. Radioactivity in 200 μl was estimated in a β counter.

Mitomycin-C Treatment

Cells were treated for 2 h with mitomycin-C (Sigma Chem. Co.) at a final concentration of 20 μg/ml. In pilot experiments, this concentration inhibited thymidine incorporation to <1% of respective controls without producing toxic effects. Monolayers were then washed three times with PBS, incubated for 30 min with fresh medium, washed again, and then trypsinized and reseeded for differentiation induction. Except for different mitomycin-C concentrations, the same procedure was previously used to demonstrate that differentiation occurs in absence of cell division in myoblasts (Falcone et al., 1984), macrophages (Beug et al., 1987), and erythroid cells (Beug et al., 1992).
Immunoprecipitation

Detection of erbA protein (p46-erbA and p55v-erbA) by [35S]methionine labeling and immunoprecipitation analysis was done as described earlier (Goldberg et al., 1988; Gilmore et al., 1990) using an anti-v-erbA antibody generously donated by Dr. J. Ghysdael.

Hormone Binding Assay

T3-specific binding assays in vivo were performed as previously described (Sap et al., 1986; Muñoz et al., 1990). Specific binding is defined as the ratio of iodinated T3 bound in absence and presence of a 1,000-fold molar excess unlabeled T3.

Detection of Neuronal and Chromaffin Differentiation Antigens by Immunofluorescence

Fixed and permeabilized cells were analyzed by indirect immunofluorescence using monoclonal antibodies to synaptophysin, tyrosine hydroxylase, glial fibrillary acidic protein (all from Boehringer Mannheim Corp., Germany), and N-CAM (Sigma Chem. Co.) according to standard procedures. Rabbit antisera to mouse E-cadherin (uromorulin; generously provided by Dr. R. Kemler) and mouse 45-kd cytokeratin (provided by Dr. E. Reichman) were used similarly. Basically, cells were fixed with 3.7% paraformaldehyde plus 0.01% glutaraldehyde at 4°C for 15 min, and then immunoprecipitated with 0.1% NP-40 in medium for an additional 15 min at room temperature. Antibodies were used at the dilutions recommended by the manufacturers in a final volume of 50 µl. After a 1-h incubation at room temperature, the plates were washed with PBS and stained with secondary antibody (FITC-conjugated anti-mouse or anti-rabbit IgG, Amersham, diluted 1:50) for 1.5 h. After staining, cells were washed three times with PBS, mounted in Mowiol (HOECHST), and viewed using a Zeiss Axioshot fluorescence microscope with epillumination.

RNA Preparation and Northern Analysis

Total RNA was prepared using the guanidinium isothiocyanate-phenol-chloroform procedure (Chomczynski and Sacchi, 1987). Purification of poly(A)+ RNA was done as described (Vennström and Bishop, 1982). Northern blots were performed on Gene Screen membranes (New England Nuclear, Boston, MA) according to standard protocols (Maniatis et al., 1982). All probes were labeled by the random priming method (Feinberg and Vogelstein, 1983) using commercial kits (Pharmacia LKB Biotechnology Inc., Piscataway, NJ). Source of probes: rat c-erbA/TRα-1 from Dr. R. Evans, rat TRβ from Dr. H. C. Towle, mouse c-jun, junD, and junB and human c-jun, c-junD, and c-junB from Dr. R. Bravo, rat p53 from Dr. T. Curran, rat NGF-R and NGF-E-B from Dr. J. Milbrandt, v-fos from Dr. E. Wagner, rat gastrin-releasing protein (GRP) from Dr. R. Breathnach, mouse collagen al (I) from Dr. Z. Q. Wang, rat SSCP10 from Dr. J. Anderson, rat GAP-43 from Dr. M. Fishman, rat tyrosine hydroxylase from Dr. E. Ziff, rat chromogranin B from Drs. S. Forss-Petter/J.G. Sutcliffe, mouse N-CAM from Dr. C. Goridis, mouse cytokeratin (endoβ/K18) from Dr. M. Busslinger, human trk from Dr. M. Barbacid, mouse trk from Dr. L. Parada, rat LNGFR from Dr. E. M. Shooter, human RARα and mouse RARβ from Dr. H. Stunnenberg, and rat NF68 from Dr. F. Grosfeld. Rat MASH-1 and MASH-2 genes were cloned from poly (A)+ RNA prepared from dense cultures of PC12 6c cells by cDNA synthesis and PCR amplification using primers specific for the extremes of the published sequences (Johnson et al., 1990) obtained from the EMBL data bank. The oligonucleotides used were 5'AAATATAATGAACTTGTCGCGTCTCCCTCTTTTAA3' 5'TATGATCAAGAA-TTCCATCTTCAAGTCCTAAA3' 5'ATGATCAGAAGATTCATCATTCCCTCTCCTCA3' 5'ATGATCAGAGACGCCTTAAAGC3' 5'TATGATCAGAGACGCTTTAAGC3' for MASH-2. The amplified products were cloned into the HindIII and EcoRI sites of the pRK7 plasmid. Fragments covering the nonhomologous 3' ends were prepared as specific probes for both genes. A 308-bp PvuII fragment was prepared for MASH-1 and a 397-bp Pst-I-ScaI fragment for MASH-2. Hybridizations were carried out overnight at 65°C in 7% SDS, 500 mM sodium phosphate buffer, pH 7.2, and 1 mM EDTA according to Church and Gilbert (1984). Filters were washed twice for 30 min each in 1% SDS and 40 mM sodium phosphate buffer, pH 7.2, at 65°C. Before rehybridizing the nylon membranes with probes for other genes, the radioactive probe was stripped off the membrane by placing it in a 75°C water bath for 5 min.

Results

Stable Expression of Exogenous erbA Genes in PC12 Cells

To analyze the possible role of T3 and its receptor on PC12 differentiation in vitro, we sought to stably express the avian c-erbA/TRα-1 protein as well as its oncogenic variant, v-erbA, in these cells. Cultures of a clonal PC12 subline (6c, see Materials and Methods) were infected with retroviruses expressing c-erbA/TRα-1 or v-erbA together with the neo gene as a selectable marker. Several neoresistant clones of c-erbA/TRα-1 infected cells were obtained. In contrast, we only succeeded to expand a pool of v-erbA-infected cell clones due to their poor clonal proliferation ability.

The expression of the respective TRα-1 and v-erbA proteins was monitored by immunoprecipitation using anti-erbA antisera. c-erbA/TRα-1 protein was just detectable, whereas v-erbA was expressed at high levels, comparable to those found in avian erythroleukemic cells (Fig. 1 A). Synthesis of active c-erbA/TRα-1 protein was confirmed by a hormone binding assay after incubation with 125I-labeled T3. Specific binding was one order of magnitude higher in c-erbA/TRα-1-expressing cells than in uninfected or v-erbA-expressing cells (Fig. 1 B), and of the same order of magnitude as that of rat GHI pituitary cells containing a few thousand active receptor molecules per cell (Samuels et al., 1976, 1977).

Endogenous Hormone Receptor Expression in Normal and erbA-expressing PC12 Cells

Since several cell lines of neural origin express the thyroid hormone receptor variant TRα-2 in high excess over TRα-1 (Muñoz, A., J. Bernal, and A. Rodríguez-Pefia, unpublished observations), we tested if this was also true for PC12. As shown by Northern blot analyses, normal PC12 cells expressed much higher levels of the TRα-2 form than of the TRα-1 and TRβ receptors (Fig. 1 C). In contrast, the retrovirus transduced exogenous c-erbA/TRα-1 and v-erbA genes were expressed at levels comparable to those of the endogenous TRα-2 receptor. Thus, the nonresponsiveness of PC12 cells to T3 may in fact be due to functional suppression of the low TRα-1 levels by an excess of TRα-2.

To rule out that the c-erbA/TRα-1- and v-erbA-expressing PC12 sublines were altered in their expression of other important receptors, the same blots were hybridized with cDNA probes encoding the low (gp80NGFR) and high (gp140*) affinity NGF receptors as well as the retinoic acid receptors (RARs) α and β. All these receptors were expressed in the parental cells (distinct signal from 10 µg total RNA after 5-d exposure) and no changes in expression levels were detected in the sublines expressing exogenous erbA proteins (data not shown).

Ligand-activated TRα-1 Renders PC12 Cells NGF-dependent for Proliferation and Survival

Neurotrophic factors such as NGF are considered to be necessary for survival of differentiating neuronal cells, including primary adrenal medulla sympathetic neurons in culture. (Doupe et al., 1985b; Anderson and Axel, 1986). In contrast, PC12 cells grow in the absence of NGF, and revert to an undifferentiated state if the factor is withdrawn af-
Figure 1. ErbA expression in PC12 cells. (A) Uninfected, TRα-1/c-erbA- and v-erbA-infected PC12 cells were labeled with [35S]methionine and lysates from equal numbers of cells were immunoprecipitated with an antibody against v-erbA. The immunoprecipitates were analyzed on 10% SDS-polyacrylamide gels and fluorographed. Films were exposed for 6 d. Bands corresponding to the chicken p46 c-erbA and the viral p75 v-erbA are indicated by arrows. The faint p46 c-erbA band is also indicated by a solid triangle to distinguish it from two bands precipitated nonspecifically (open triangles). (N) preimmune serum; (I) immune serum. (B) Uninfected and infected PC12 cells were analyzed for T3-specific binding as described (Sap et al., 1986; Muñoz et al., 1990), in presence (+T3) or absence (-T3) of a 1,000-fold molar excess (0.3 μM) of cold T3 as competitor. Rat pituitary GH1 cells expressing high levels of endogenous T3 receptors were used as a control. (C) Analysis of TR RNA expression by Northern blots in uninfected, TRα-1- and v-erbA-infected PC12 cells. 10 μg of total RNA were analyzed per lane. Exposure times: rat c-erbA/TRα, 6 d; rat c-erbA/TRβ, 14 d. Mature endogenous TRα-1 RNA (lower band, 5 kb) and its precursor (upper band, 6.5 kb) are indicated with asterisks (*). The band corresponding to the endogenous TRα-2 RNA (2.6 kb) is marked with an (x). It coincides with that of the viral subgenomic RNA encoded by the internal promoter. This RNA, as well as the large unspliced viral genomic RNAs (indicated by arrows) crosshybridize with both rat c-erbA probes, α and β. Hybridization with the β probe gives two very weak signals corresponding to the precursor (6.5 kb, arrowhead) and mature (4 kb) RNAs.

ter induction of differentiation. To determine whether TRα-1 expression would affect PC12 cells responsiveness to NGF, we first studied the effects of T3, NGF, or their combination on the capacity of uninfected and c-erbA expressing cells to proliferate and to synthesize DNA.

As shown in Fig. 2, treatment with NGF alone or NGF plus T3 did not grossly affect proliferation of uninfected cells (panel A, left). In cells expressing exogenous TRα-1 however, T3 and NGF had clear but opposite effects on survival and proliferation. NGF alone led to a drastic increase in proliferation, as well as a dramatic decrease in cell viability. T3 alone had little effect on proliferation, but led to a marked decrease in cell viability. The combination of T3 and NGF led to a dramatic increase in cell viability and a moderate increase in proliferation. These results suggest that T3 and NGF have opposing effects on PC12 cell proliferation and survival.

I-PC12 cells were incubated with mitomycin C (Mit. C, see Materials and Methods) or left untreated (Control). They were then incubated with NGF for 6 d (+ NGF) or with NGF-free medium (no NGF). Phase micrographs of these cells (middle and lower panels) as well as of respective uninfected PC12 cells induced to differentiate with NGF are shown (upper panels). Bar, 50 μm.
in the level of DNA synthesis (Fig. 2, panel A, middle) which was reflected by a 30% increase in cell number after 5 d (not shown). T3 had an opposite effect: DNA synthesis ceased, and cells no longer divided and degenerated progressively. None of these effects were observed upon combined treatment with NGF and T3.

These observations suggested that ligand-activated TRø-1 indeed rendered PC12 cells dependent for survival and proliferation on neurotrophic factors like NGF. To confirm this, cells were pretreated for one week with NGF + T3, and then cultured in medium containing no additions of either T3 or NGF separately. When switched to T3 containing medium, more than 80% of TRø-1 cells degenerated within 3 d (Fig. 2 B), while they survived as differentiated cells in control cultures containing NGF + T3 (see below). A significant proportion of TRø-1 cells died even in plain medium and only NGF was able to prevent cell death. V-erbA-expressing cells were completely refractory to any of these treatments (Fig. 2 B), while normal, uninfected cells showed weak, intermediate responses (data not shown). Whether or not the cells died by programmed cell death (apoptosis) after removal of NGF as shown to take place during normal CNS development in vivo (for review see Oppenheim, 1991) is still under investigation.

C-erbA/TRø-1 and v-erbA Affect NGF-induced Neuronal Differentiation in PC12 cells

To determine whether TRø-1 or v-erbA expression affects the ability of PC12 cells to differentiate into neurons in response to NGF, uninfected cells, and cells expressing c-erbA/TRø-1 or v-erbA were treated with NGF, T3, or both for 5–7 d. The extent of neuronal differentiation was estimated by monitoring neurite outgrowth and expression of two neuronal markers, synaptophysin and N-CAM (Prentice et al., 1987; Mann et al., 1989), on neurites and cell bodies by immunofluorescence. As controls, cells were stained with antibodies to tyrosine hydroxylase (TH), an enzyme which is downregulated by NGF but induced by dexamethasone during chromaffin differentiation (Lewis et al., 1983; Leonard et al., 1987; Stein et al., 1988b), and to glial fibrillary acidic protein, an astrocyte marker.

As expected, NGF treatment of uninfected cells induced cell flattening followed by appearance and progressive elongation of neurites and rounding of cell bodies (Fig. 3). The neurites expressed high levels of synaptophysin and lower levels of N-CAM, while TH expression decreased (from 55 to 16% fluorescence positive cells; Fig. 3). As mentioned above, T3 alone had no major effects on normal PC12 cells and did not detectably alter NGF-induced differentiation, when applied together with NGF (data not shown).

In contrast, expression of exogenous TRø-1 strongly arrested NGF-induced differentiation. When treated for 6 d with NGF alone, the cells developed only short processes that failed to elongate even after prolonged NGF application (Fig. 4). TRø-1-cells also failed to upregulate synaptophysin and N-CAM expression in response to NGF (Fig. 4 and data not shown). TH content was low already in the uninduced stage (20% positive cells) and was further reduced after NGF (2.5% positive cells, not shown).

Interestingly, the differentiation block could be completely reversed by addition of T3 together with NGF. After 6 d of exposure to both agents, a high proportion of TRø-1 cells produced long processes expressing synaptophysin and N-CAM at similar or even higher levels as uninfected cells in the presence of NGF alone (compare Figs. 3 and 4, data not shown). In the absence of NGF, T3 induced progressive cell death which affected 30–80% of the population after 5 d depending
on the clone analyzed (Fig. 4, lower panels and data not shown).

Cells expressing v-erbA showed a distinctly different response. They were arrested in neuronal differentiation regardless of whether the cells were treated with NGF alone or in combination with T3 (Fig. 4). In both cases, they formed only short processes that expressed no synaptophysin with NGF alone, while some expression was seen at the neurite tips with NGF + T3 (Fig. 4). This latter result agrees with the assumed role of v-erbA as a constitutive repressor of TR-regulated genes (Sap et al., 1989; Damm et al., 1989). T3 alone had no effect on these cells.

Since cells expressing TRa-1 were induced by NGF to grow at an increased rate we had to rule out that their differentiation arrest was a consequence of an increased proliferation and cell density. To rule out this possibility cell differentiation was studied after inhibition of cell proliferation with mitomycin-C. Uninfected PC12 cells differentiated normally in response to NGF in presence or absence of mitomycin-C (Fig. 2 C, upper panels). In contrast, both mitomycin-treated and control TRc~l cells formed only short processes in response to NGF (Fig. 2 C). This suggests that the stimulation of proliferation and the differentiation arrest caused by TRa-1 in NGF-treated PC12 cells are unrelated phenomena.

Retinoic Acid Receptors Do Not Play a Major Role in TRa-1 Regulated Neuronal Differentiation of PC12 Cells

Since PC12 cells express retinoic acid receptors (RARα and RARβ) and cooperation of TRs and RARs is thought to be crucial for thyroid hormone action in several systems (Hudson et al., 1990; Schroeder et al., 1992b), we determined whether retinoic acid (RA) would affect T3-dependent regulation of differentiation in normal and TRα-1-expressing PC12 cells. In either cell type, RA did not grossly affect neuronal differentiation induced by NGF or NGF + T3. Also, RA did not alter the T3-induced NGF dependence of TRα-1 cells. However, if used alone, RA seemed to induce cell rounding and formation of aggregates similar to those seen during the initial stages of dexamethasone-induced chro-

Figure 4. Arrest of NGF-induced neuronal differentiation by TRα-1 and v-erbA. Phase micrographs of TRα-1 and v-erbA-expressing PC12 cells treated with NGF, NGF plus T3 (7 d) or T3 alone (5 d), or left untreated (Control) are shown. Middle panels (TRα-1) and insets to right panels (v-erbA) show the corresponding immunofluorescence analysis using synaptophysin antibody (see Materials and Methods). Bar, 50 μm.
maffen differentiation (data not shown). Again, this response was not affected by the presence or absence of exogenous TRα-1, suggesting that the RARs do not play a major role in the regulation of neuronal differentiation by TRα-1.

TRα-1 Does Not Affect the Induction of Early Genes by NGF

Several dozen genes are known to be induced by NGF in PC12 cells (for a review see Halegoua et al., 1991). These genes can be distributed in two groups: early response genes whose induction begin a few minutes after NGF addition and peak one or a few hours later, and late response genes which are induced with slower kinetics and reach their maximum level of expression two, four, or more days after continuous NGF treatment.

First, we analyzed possible effects of TRα-1 on the expression of NGF-inducible early genes, such as c-jun, jun-B, jun-D, c-fos, fra-1, NGFI-A (Krox 24, egr-1, zif/268), and NGFI-B (nur/77, N10) which have been reported to be induced by NGF to a variable extent (Greenberg et al., 1985; Kruijer et al., 1985; Morgan and Curran, 1986; Milbrandt, 1987, 1988; Bartel et al., 1989; Szeberényi et al., 1990). The fra-1 gene was particularly interesting, since its constitutive expression apparently blocks induction of many of the other early genes (Ito et al., 1990). Uninfected and TRα-1-expressing cells were treated for 1 h with NGF, T3, or both hormones, and subjected to Northern blot analysis, using poly(A)+ RNA (Fig. 5).

NGF caused a similar transient upregulation of all these genes in uninfected and TRα-1-expressing cells with strong increments of mRNA levels after 1 h, and much less after 24 h. Furthermore, T3 neither superinduced nor suppressed the induction of any of these genes by NGF in either cell type.
Similar patterns were obtained for ornithine decarboxylase (not shown). These results show that TRα-1 does not interfere at all with NGF-regulated early gene expression, indicating that NGF is able to induce all its normal intracellular signals in TRα-1-expressing PC12 cells.

**TRα-1 Causes Hormone-dependent Repression of NGF-induced Late Genes**

Having ruled out that TRα-1 affects NGF-dependent signal transduction, we checked the alternative hypothesis that TRα-1 might block the expression of neuronal-specific genes in absence of ligand, but allow or even enhance their NGF-regulated expression in presence of T3. Such a result would be expected from a similar behavior of TRα-1 in transient transfection experiments (Damm et al., 1989; Sap et al., 1989) as well as in avian erythroblasts (Zenke et al., 1988, 1990). Consequently, cells were treated with NGF alone, NGF + T3, or left untreated, and tested for late gene expression in Northern blots.

TRα-1 strongly affected expression of a large series of NGF-inducible late genes (Fig. 6). This group consisted of...
four neuron-specific genes (SCG10, neurofilament 68, N-CAM, and GAP-43; Prentice et al., 1987; Stein et al., 1988a,b; Lindenbaum et al., 1988; Federoff et al., 1988), the protease transin probably involved in neurite outgrowth (Machida et al., 1989) and extracellular matrix proteins and their receptors (collagen-α1 [I], integrin-β1; Mann et al., 1989; Van Hoof et al., 1989; Costello et al., 1990). In absence of T₁, TRα-1 efficiently repressed upregulation of all these genes by NGF. Exposure of the cells to NGF plus T₁ stimulated expression of all genes to similar or even higher levels than observed in normal PC12 cells induced to differentiate by NGF alone or by NGF + T₁. In addition, several of these genes (e.g., SCG-10, integrin-β1) appeared to be repressed in PC12 cells expressing unliganded TRα-1 (Fig. 6). A similar pattern was found for two additional genes (peripherin and MAP-2) that were only weakly induced by NGF (data not shown). Thus, TRα-1 regulates neuronal gene expression in a fashion faithfully reflecting its effect on morphological differentiation.

**TRα-1 Does Not Affect NGF-induced Repression of the Early Neuroblast Marker MASH-1**

The MASH-1 and MASH-2 genes, rat homologues of the Drosophila achaete-scute genes probably involved in neuronal development, were recently isolated as candidates for neuronal "master" genes (Johnson et al., 1990). Later work suggested that the MASH-1 gene may be a marker for early neuronal progenitors and that NGF causes the loss of this early marker in PC12 cells (Lo et al., 1991). Because of their possible significance for neuronal differentiation, it was of interest to study if MASH gene expression was regulated by TRα-1.

Northern blot analysis revealed no detectable expression of the MASH-2 gene (not shown). MASH-1 mRNA levels remained unchanged in normal and TRα-1 cells after 1 h treatment with NGF or NGF + T₁ (Fig. 6). Treatment for 24 or 144 h with these agents caused a significant decrease in MASH-1 mRNA levels in both cell types, although down-regulation of MASH-1 message by NGF alone occurred more slowly in the TRα-1-expressing cells. These findings agree with those from Lo et al. (1991) and confirm our results on the early NGF-induced genes, suggesting that TRα-1 does not simply block NGF action, but causes a late arrest in PC12 differentiation via an NGF-independent pathway.

**TRα-1 Prevents Entry of PC12 Cells into the Chromaffin Differentiation Pathway**

Having shown that TRα-1 regulated neuronal differentiation and gene expression in a hormone-dependent fashion, we next investigated whether TRα-1 might affect the alternative differentiation pathway open to PC12 cells, i.e., chromaffin differentiation induced by dexamethasone (DEX). DEX was reported to cause morphological changes and expression of some chromaffin markers in PC12 cells (Greene and Tischler, 1982) and to antagonize the action of NGF at the molecular level (Leonard et al., 1987; Stein et al., 1988b).

After prolonged DEX treatment (20 d), uninfected PC12 cells adopted a rounded or polygonal shape, strongly adhering to each other, and formed cords and clumps which eventually fused into compacted epithelioid islands. This phenotype was not altered by the presence of T₁. In contrast, DEX-treated TRα-1 cells showed a delay in the formation of cell clumps which failed to develop into dense, compact islands. In presence of T₁, the cultures contained even more individual cells and few loose clumps, cells displaying a polygonal shape and short neurite-like spikes (data not shown).

These pilot studies raised the possibility that TRα-1 inhibited DEX-induced differentiation of PC12 cells into epithelioid, chromaffin-like cells. This was confirmed by the pattern of expression of epithelial markers such as cytokeratins and E-cadherin (uvomorulin). Strong expression of cytokeratins and weak, but distinct expression of membrane-bound uvomorulin was seen in the epithelioid islands obtained from DEX-treated uninfected cells. Expression of both markers was essentially missing in the loose groups of TRα-1-expressing cells subjected to the same treatment (data not shown, see below). We also studied the expression of the chromaffin marker TH. As expected, TH was strongly up-regulated by DEX in uninfected cells (55–98% strongly positive cells, Fig. 7A). In contrast, TRα-1 cells expressed only low levels of TH (<10% weakly positive cells), which were only weakly upregulated by DEX (<30% positive cells; Fig. 7A).

Differentiation of uninfected PC12 cells induced by prolonged DEX treatment was irreversible (Fig. 7B). Even after DEX removal and subsequent treatment with NGF or NGF + T₁, most cells remained forming epithelioid islands expressing chromaffin markers (cytokeratin and uvomorulin). In contrast, TRα-1 expression prevented irreversible chromaffin differentiation. TRα-1 cells treated sequentially with DEX and NGF appeared immature with numerous short neurites and failed to express uvomorulin or cytokeratin (except for a low percentage of cytokeratin-positive cells; Fig. 7B). As expected, if T₁ was administered together with NGF the cells differentiated into neurons. These results indicate that TRα-1-expressing PC12 cells respond abnormally to DEX induction. Instead of differentiating into chromaffin cells, they retain an undifferentiated phenotype and remain inducible by NGF + T₁ to form neurons. Thus TRα-1 constitutively prevents entry of PC12 cells into the chromaffin pathway, raising the possibility that TRα-1 expression commits the cells to the neuronal pathway of differentiation.

The effect of TRα-1 in blocking entry into the chromaffin pathway was also confirmed by studying the expression of two genes, TH and chromogranin B which although expressed in neurons are induced by DEX during chromaffin differentiation of PC12 cells (Lewis et al., 1983; Leonard et al., 1987; Stein et al., 1988b). Both basal and DEX-induced expression of TH was blocked in cells expressing TRα-1 (Figs. 6 and 8). Neither NGF nor T₁ or both could release the suppression of TH mRNA levels (Fig. 6). A much weaker suppressive effect of TRα-1 was observed for the chromogranin B and cytokeratin genes.

**V-erbA Constitutively Induces an Aberrant Phenotype Combining Neuronal and Chromaffin Characteristics**

The v-erbA oncogene acts as a constitutive repressor of red cell differentiation and erythrocyte gene expression in avian erythroblasts (Zenke et al., 1988, 1990; Disela et al., 1991). It also interferes with transactivation of synthetic TR/RAR...
Figure 7. Effects of TRα-1 and v-erbA on dexamethasone-induced chromaffin differentiation in PC12 cells. (A) Immunofluorescence analysis of tyrosine hydroxylase protein expression in uninfected, TRα-1-, and v-erbA PC12 cells treated with dexamethasone for 2 wk (DEX) or left untreated (Control). Bar, 50 μm. (B) (Upper group of panels; Morphology) Uninfected PC12 cells and TRα-1-PC12 cells were treated for 20 d with dexamethasone. They were then washed and incubated for an additional 7 d with NGF (Dex~NGF) or NGF + T3 (DEX + NGF + T3). Phase micrographs of representative areas are shown. Note presence of dense epithelioid islands surrounded by neuron-like cells in the normal PC12 cells. Bar, 50 μm. (Lower group of panels) Immunofluorescence analysis on parallel dishes to those photographed in the upper panels, using uvomorulin (E-cadherin) and cytokeratin antibodies, is shown.

response elements by RARα (Sharif and Privalsky, 1991) and has been claimed to prevent inhibition of fibroblast proliferation by RA (Desbois et al., 1991). We therefore studied in more detail how v-erbA affected differentiation and gene expression in PC12 cells.

As already described above, v-erbA expression caused a constitutive, partial inhibition of NGF-induced neuronal differentiation, regardless of the presence or absence of thyroid hormone (Fig. 3). In contrast to TRα-1 cells, DNA synthesis and cell proliferation were not affected by NGF or T3 treatment in v-erbA cells (Fig. 4 A). Most strikingly, T3 did not induce an NGF dependence for cell survival, and even the weak cytopathic effect induced by T3 alone in uninfected cells was completely absent in v-erbA expressing cells (Fig. 4 B). This protective effect of v-erbA may well be due to suppression of endogenous c-erbA action.

Similarly to TRα-1, v-erbA did not affect the induction of early genes by NGF (Fig. 5). In contrast to TRα-1, however, v-erbA did not cause a repression of most late NGF inducible genes studied (Fig. 6) and was not able to prevent their induction by NGF. As expected from the inability of the v-erbA protein to bind and respond to hormone (Sap et al., 1986; Muñoz et al., 1988, 1990, see Fig. 1), T3 did not alter expression levels of most NGF-inducible genes in v-erbA cells.

The transin (stromelysin) gene was an interesting exception. Induction of this gene by NGF was strongly inhibited in v-erbA cells as compared to normal PC12 cells (Fig. 6). This repression persisted even after incubation with NGF + T3, leading to much lower levels of transin mRNA than obtained in normal or c-erbA cells treated similarly. It seems possible, therefore, that v-erbA is able to constitutively repress a subset of NGF-inducible genes, in line with its ability to partially inhibit neuronal differentiation.

Finally, v-erbA had an unexpected effect on DEX-induced chromaffin differentiation and gene expression. Treatment of v-erbA cells with DEX caused their flattening and aggregation into small groups, but epithelioid islands were not observed even after prolonged DEX treatment. In line with this finding, no E-cadherin could be detected on the cell membranes by immunofluorescence (data not shown). However, the cells expressed constitutively elevated levels of cytokeratin and TH. Even in untreated cells, staining with TH and cytokeratin antibodies was very strong (Fig. 7 A and data not shown) and even increased after DEX treatment (Fig. 7 A).
The results obtained for chromaffin marker gene expression were in line with these observations. Untreated or NGF-treated v-erbA cells expressed significantly higher levels of TH, chromogranin B, and cytokeratin messages than the respective uninfected cells (Fig. 8). However, DEX was still able to further induce these genes to similar levels in both normal and v-erbA-expressing cells. This suggests that v-erbA constitutively upregulates chromaffin marker gene expression, but does not prevent their regulation by DEX (Fig. 8).

A summary of the effects of c-erbA/TRα-1 and v-erbA on PC12 cell differentiation and survival is represented in Fig. 9. To rule out the trivial possibility that our v-erbA-PC12 cells fail to differentiate because of irreversible cell line progression changes, we tried to induce differentiation in...
these cells by an alternative pathway. Cyclic AMP has been shown to induce part but not all of the NGF-induced phenotypic and gene expression changes in PC12 cells, most likely via pathway(s) independent of those important for NGF action (see Halegoua et al., 1991). Indeed, NGF plus dibutyryl-cAMP (0.1 mM) induced distinct morphological differentiation characterized by typical long neurites in both TRα-1- and v-erbA-expressing PC12 cells. In line with published work (Gunning et al., 1981), dibutyryl cyclic AMP alone at high concentrations (1 mM) also induced partial differentiation in both cell types. Thus, the v-erbA-induced differentiation arrest cannot be due to mutational changes in our cell lines.

Spontaneously Immortalized Rat Brain Neuroblasts Differentiate in Response to T3 and NGF after Introduction of an Exogenous TRα-1 Gene

Since TRα-1 has no reported function in differentiation of normal adrenal medulla progenitors, the possibility remained that our results employing the PC12 cell system represented the aberrant behavior of a tumor cell line, mimicking normal differentiation processes as a consequence of artificial experimental treatments. We therefore sought to demonstrate an effect of TRα-1 in neuroblasts derived from the CNS, where the involvement of thyroid hormone receptors in neuronal maturation is amply documented (see introduction). Consequently, we introduced the avian TRα-1 into the E 18 cell line, which has been obtained by spontaneous immortalization from cultures of 17-d rat embryonic brain (Seliger, B., unpublished observations). E 18 cells represent primitive neuroblasts that express NF 68 and the primitive neuronal marker nestin, but lack the astrocyte marker, glial fibrillary acidic protein. After partial differentiation induction with dibutyryl-cAMP, the cells express further neuronal markers such as NF 145, NF 220, and neuron-specific enolase. Similarly to PC12 cells, the E18 cells expressed only low levels of TRα-1 together with high levels of TRα-2 (data not shown).

To test the ability of TRα-1 expressing E 18 cells to differentiate in response to NGF, T3, or both, we used an E 18 clone infected with the TRα-1 retrovirus as described for PC12 cells. This clone expressed TRα-1 at similar levels as the TRα-1 PC12 clone cA4 (data not shown). As shown in Fig. 10, TRα-1 E 18 cells failed to differentiate in response to NGF alone, a property shared with uninfected E 18 cells. Also, NGF seemed not to influence E 18 cell proliferation. Upon T3 addition, cells became less adherent and partially disintegrated (Fig. 10, T3) mirroring the cell killing effect observed in PC12 cells. In presence of both NGF and T3, however, essentially all cells underwent massive neuronal differentiation characterized by outgrowth of long neurites.

Figure 10. T3 plus NGF induce differentiation in TRα-1 expressing E 18 neuroblasts derived from embryonic rat brain. Phase micrographs are shown from E 18 cA9 cells cultivated in serum-free differentiation medium for 6 d in absence (Control) or presence of T3, NGF, or both (NGF + T3). Note effective differentiation into neuron-like cells upon treatment with both NGF and T3 (panel NGF + T3) Inset: Some of the neuron-like cells obtained with NGF + T3 in a separate experiment at higher magnification. Note also cell disintegration (refractile small bodies) in the T3-treated cultures. Bar, 50 μm. Inset: bar, 20 μm.
within 6 d, often in a bipolar fashion (Fig. 10). Thus, TRα-1 expression in CNS derived neuroblasts conferred responsiveness to T3 in a fashion very similar to that observed in TRα-1 expressing PC12 cells.

Discussion

Effect of TRα-1 on PC12 Cell Differentiation: Evidence for a NGF-independent, Permissive Function in Neuronal Differentiation

In this study, we provide the first direct evidence that TRα-1 controls neuronal differentiation and expression of neuron-specific genes in PC12 cells. Both NGF-induced neuronal differentiation and expression of late genes are arrested by TRα-1 in the absence of its ligand, T3, while the block is released in the presence of the hormone, leading to efficient cell maturation (see Fig. 9 for a model). These effects of TRα-1 are strikingly similar to those observed in avian erythroblasts where TRα-1 regulates erythroid differentiation and erythrocyte gene expression in a T3-dependent fashion (Zenke et al., 1990; Disela et al., 1991).

How does unliganded TRα-1 arrest neuronal differentiation? First, its action seems to be independent of NGF, since it did not affect expression of NGF-induced early genes. It also failed to inhibit NGF action completely since NGF-treated TRα-1-PC12 cells formed short neurites and lost expression of the early neuronal marker MASH-1. Rather, TRα-1 probably causes a late block in neuronal differentiation, perhaps by repressing or limiting the expression of indispensable, neuron-specific proteins. A possible candidate for such proteins is N-CAM, whose expression correlates very well with morphological differentiation of PC12 cells and causes neurite formation if expressed artificially in non-neuronal cells (Mann et al., 1989; Doherty et al., 1991). However, it is more likely that the observed block of differentiation is a combined result of the insufficient expression of many proteins with such diverse functions as maintenance of neurite structure or function and regulation of cell motility or cell-substrate adhesion.

The second important effect of TRα-1 was its ability to render differentiating PC12 cells dependent on NGF for survival. These results may correspond to the well-known fact that primary neurons differentiating in culture require neurotrophic factors for survival and neurite outgrowth. By such a mechanism, TRα-1 could control naturally occurring neuronal death during vertebrate development where large percentages (up to 80%) of certain neuronal populations undergo programmed cell death (Oppenheim, 1991). In line with this idea, thyroid hormone controls tissue regression and neuronal death during amphibian metamorphosis (Kollros, 1981; Tata, 1984; Tata et al., 1991) and the survival of cerebellar granule cells in hypothyroid rats (Lewis et al., 1976).

The effects of TRα-1 and thyroid hormone were not limited to PC12 cells. Studies on the E 18 cell line suggest that similar mechanisms are likely to be operating in vivo in selected neuronal populations of the CNS. In line with these findings we propose that TRα-1 controls neuronal differentiation in a permissive fashion: Neuronal progenitor cells are arrested at a certain stage of maturation by nonliganded TRα-1, until they are released to differentiate when ligand becomes available. At this stage, neurotrophic factors like NGF are required for survival of the differentiating cells. Thus, locally produced neurotrophic factors (NGF) and the systemic endocrine agent T3 seem to cooperate in optimal induction of neuronal differentiation and neuronal gene expression. Cooperation of thyroid hormone and NGF has been demonstrated in vivo during development of the hippocampus, olfactory bulb, and cerebellum (Clos and Legrand, 1990), regions which express abundantly the TRα isoform of T3 receptor (Mellström et al., 1991).

In contrast to the erythroblast system (Schroeder et al., 1992a,b), TRα-1 does not seem to cooperate with RARs in neuronal differentiation of PC12 cells since the respective ligand (RA) did not affect this process or its regulation by TRα-1. Whether such a cooperation would occur upon artificial expression of RARs via retrovirus vectors or in mutant PC12 cells expressing elevated RAR levels (Scheibe et al., 1991) remains to be determined. The RARs might, however, be involved in regulating chromaffin differentiation, since RA induced morphological changes in PC12 cells resembling those induced by DEX. If this was true, the RARs might antagonize the function of TRα-1 rather than cooperating with it.

V-erbA Acts Like a Weak Constitutive Repressor of NGF-induced Differentiation and Gene Expression

In contrast to TRα-1, the v-erbA oncoprotein caused a partial, but constitutive block of NGF-induced PC12 cell differentiation. With the exception of the transin gene, expression of which was constitutively repressed by v-erbA, NGF regulation of other late genes studied was hardly affected by v-erbA. This could be due to the fact that v-erbA has a lower binding affinity to regulatory sequences of target genes (Sap et al., 1989; Disela et al., 1991). In addition, its expression level in PC12 cells was perhaps not high enough to efficiently repress the genes studied other than transin. Even in avian erythroblasts (the cell type in which v-erbA has been selected for its oncogenic activity) a large excess of v-erbA is necessary to repress genes activated by TRα-1/c-erbA or RARS (Disela et al., 1991; Schroeder et al., 1992a).

Our results suggest that v-erbA constitutively affects both lineages of PC12 cell differentiation. It partially arrests neuronal differentiation and gene expression, and also inhibits chromaffin differentiation although it is able to constitutively turn on some DEX-induced genes. As a result, v-erbA gives rise to an aberrant "interlineage" phenotype characterized by coexpression of certain neuronal and chromaffin markers (see Fig. 9 for a model). There are two nonexclusive possibilities to explain how v-erbA and TRα-1/c-erbA affect chromaffin differentiation and gene expression in such a different fashion. First, the point mutations in the v-erbA DNA binding domain might alter the DNA binding specificity of this mutated hormone receptor in that it now constitutively activates genes that are repressed by TRα-1. More likely, however, v-erbA differs from TRα-1/c-erbA in the way it interacts with other transcription factors such as RARS (Sharif and Privalsky, 1991, Desbois et al., 1991) or members of the AP-1 transcription factor family (Zhang et al., 1991) in transient transcription assays using standard cell lines. This functional difference between v-erbA and TRα-1 may both explain their different regulation of the chromaffin
marker gene tyrosine hydroxylase and their different effect on NGF-regulated proliferation of PC12 cells. Zhang et al. (1991) recently reported that T3-activated c-erbB inhibits AP-1 activity in transiently transfected cells. This may explain why T3 inhibits cell proliferation in TRα-1-PC12 cells. In addition, these authors showed that a truncated c-erbB carrying a COOH-terminal deletion very similar to that present in v-erbB was unable to block AP-1 activity, perhaps explaining why proliferation of our v-erbB-PC12 cells was not affected by T3 (Fig. 4 A).

TRα-1-Dependent Regulation of PC12 Differentiation: Relevance for Neuronal Differentiation in the CNS

Our results have several immediate consequences for the understanding of how thyroid hormone might act in the CNS. The observed permissive action of TRα-1 on neuron maturation could be an underlying mechanism for the abnormalities occurring in the hypothyroid rat brain. Unliganded TRα-1 might cause a late block in neurotrophin-dependent neuronal maturation. Similarly, the observation that the lesions can be reversed by T3 administration during a critical period (Legrand, 1984) agrees well with the fact that T3 releases the cells from this late block. By using the PC12 cell line approach, we were able to identify a good-sized number of candidate genes for regulation by T3 and its receptor. Such genes may be impossible to detect by cDNA library screening approaches employing the whole brain or parts of it (Muñoz et al., 1991), since they may only occur in selected neuronal cell populations or during specific phases of brain development.

Although this work strongly suggests a direct, important role of thyroid hormone receptors in brain development, we are ignorant about mechanisms governing TR expression. The ratios between the different TR types, and the (likely) functional differences between the different TRs. Thus, studies like those presented here will have to be extended to other, preferably brain-derived neural cell types, to other TR types, and to the other neurotrophic factors recently identified (Barde et al., 1982; Leibrock et al., 1989; Lin et al., 1989; Hohn et al., 1990; Jones and Reichardt, 1990; Maisonnier et al., 1990; Berkemeier et al., 1991; Hallböök et al., 1991). An obvious choice for such systems will be neuroblast lines such as the E 18 line described here. Due to the instability of such lines and their reported ability to change their phenotype (e.g., from neuronal to glial, Gage, 1992), more work is required before such lines can be used for molecular studies in the fashion described here for the PC12 system.

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