Involvement of T-complex Protein-1Δ in Dopamine Triggered Apoptosis in Chick Embryo Sympathetic Neurons*

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The neurotransmitter dopamine (DA) is capable of inducing apoptosis in post-mitotic sympathetic neurons via its oxidative metabolites. The differential display method was applied to cultured sympathetic neurons in an effort to detect genes whose expression is transcriptionally regulated during the early stages of DA-triggered apoptosis. One of the up-regulated genes was identified as the chick homologue to T-complex polypeptide-1Δ (TCP-1Δ), a member of the molecular chaperone family of proteins. Each chaperone protein is a complex of seven to nine different subunits. A full-length clone of TCP-1Δ was isolated containing a predicted molecular weight of 57,736. Comparison with the mouse TCP-1Δ revealed 78 and 91% homology on the DNA and protein levels, respectively. Northern blot analysis disclosed a steady and significant increase in mRNA levels of TCP-1Δ after DA administration, reaching a peak between 4 and 9 h and declining thereafter. Induction of the TCP-1Δ protein levels was also observed as a function of DA treatment. Overexpression of TCP-1Δ in sympathetic neurons accelerated DA-induced apoptosis; inhibition of TCP-1Δ expression in these neurons using antisense technology significantly reduced DA-induced neuronal death. These findings suggest a functional role for TCP-1Δ as a positive mediator of DA-induced neuronal apoptosis.

Apoptosis of neuronal cells has been suggested to play a role in several neurodegenerative disorders (1–4), although the early events that move these cells toward commitment to the intrinsic death program are still unknown. Parkinson’s disease (PD)1 results from selective degeneration of the dopaminergic nigrostriatal neurons. The etiology of this process is not known, but one explanation may be enhanced oxidative stress which, in the substantia nigra, could stem from reactive oxygen species generated during normal metabolism of dopamine (DA) (5). Indeed, auto and enzymatic oxidation of DA, the endogenous nigrostriatal neurotransmitter synthesized and stored in the nigrostriatal neurons, generates a variety of cytotoxic oxygen radical species (6).

In support of this concept, we recently showed that DA can induce apoptosis in cultured neuronal as well as non-neuronal cells, including chick embryo sympathetic neurons, mouse thymocytes, and rat phaeochromacytoma cells (PC12) (7–10). Based on these findings we hypothesized that neuronal degeneration in PD may be caused at least in part by DA-mediated induction of apoptosis in the nigrostriatal neurons. Dysregulation of the cellular control mechanisms of apoptosis, which may be inherited or acquired, can lead to failure to restrain the lethal potential of DA and its potentially toxic oxidation products (11–14).

To understand the molecular events associated with neuronal degeneration, we focused on the early stages of the DA-triggered death process in cultured neuronal cells. Genes whose expression was selectively changed in response to DA treatment were demonstrated by the differential display (DD) method, which detects differences between cell populations in the mRNA repertoire (15, 16). This “non-hypothesis bound” approach, applied to cultured chick embryo sympathetic neurons, enabled us to isolate several “DA-responsive” genes, i.e. genes undergoing up- or down-regulation in response to exposure to DA (or its oxidative metabolites). Some of these genes may have a functional role in mediating the apoptosis process (17–19).

Several reasons led us to choose sympathetic neurons as a model system to study DA toxicity. (a) Similar to dopaminergic neurons, sympathetic neurons synthesize, store, and secrete catecholamines. (b) The requirement of large numbers of neurons precluded studies on the subpopulation of dopaminergic neurons, which comprise only 3% of the total neuronal population of the substantia nigra. (c) Since all the cells we investigated (several types of neurons, PC12 cells, thymocytes including dopaminergic neurons (7–10, 20)) were susceptible to the apoptotic triggering potential of DA, similar cellular mechanisms might be at work. (d) Since dopaminergic neurons synthesize, store, and secrete DA, it is feasible that under certain conditions the secreted and/or accumulated DA becomes toxic and induces apoptotic neuronal death, making it an important mediator of PD.

We report here that during DA-induced apoptosis, one of the up-regulated genes identified by DD methodology was the chick homologue of the T-complex protein-1Δ (TCP-1Δ) subunit. Chaperonin-containing TCP-1 (CCT) is a large multisubunit complex of 800–900 kDa, a protein complex considered to be...
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the cytosolic homologue of mitochondrial HSPer60. These two complexes have a characteristic oligomeric structure consisting of two heptameric rings stacked one on top of the other to form a large double-ring complex. The complexes recognize proteins in non-native conformation, thereby preventing premature folding and aggregation, and mediates the acquisition of the native structure (21). Cytosolic CCT is a highly conserved protein that interacts with only a limited number of substrates such as actin, tubulin, luciferase, and cyclin B (22, 23).

We report the cloning, sequencing, and transcript analysis of TCP-1β and show that it is up-regulated as a result of DA-induced apoptosis. Finally, we show that overexpression of TCP-1β protein accelerates DA-induced apoptosis and that down-regulation of TCP-1β by antisense treatment reduced the rate of DA-induced neuronal death.

MATERIALS AND METHODS

Cell Cultures and DA Treatment

Chick Embryo Sympathetic Neurons—Fertilized eggs were obtained from the Tel Aviv University animal care facility. Paravertebral sympathetic ganglia at embryonic day 9 (8, 24) were dissected out, and the cells were dissociated by trypsinization (0.25% in EDTA for 30 min at 37 °C). Cells were then plated on 24 poly-l-lysine-coated wells (4 × 10⁶ cells/well) or 35-mm tissue culture plates (10⁶ cells/well) (TPP, Trasadingen, Switzerland) and grown in serum-free medium DCCM-1 (Biological Industries, Kibbutz Beit Haemek, Israel) supplemented with 2% chick embryo extract, 0.5% horse serum, 20 mM, and no RNase inhibitor was added.

Specific Reverse Transcription—DD—With the exception that dNTP concentration was 1 mM, and specific primer concentration was 0.5 µM. Taq polymerase was from Bioprobe Systems, France. Cycling conditions varied according to specific primers. The identity of each PCR product was confirmed by subcloning into pGEM-T vector (Promega, Madison, WI) and sequencing the cloned insert. These inserts served as probes for Northern blot analysis.

Specific Amplification of Reverse Transcription Products—The whole volume (20 µl) of reverse transcription reaction was taken for PCR. In a final volume of 100 µl, dNTP concentration was 200 µM, and specific primer concentration was 0.5 µM. Taq polymerase was from Bioprobe Systems, France. Cycling conditions varied according to specific primers. The identity of each PCR product was confirmed by subcloning into pGEM-T vector (Promega, Madison, WI) and sequencing the cloned insert. These inserts served as probes for Northern blot analysis.

Rescue of Differentially Expressed Bands

cDNA bands that consistently showed different expression patterns were chosen for further characterization. Precise orientation between x-ray film and the dried film was achieved by fluorescent markers (Stratagene, La Jolla, CA). Bands were cut out through the film, and the gel was re-exposed to x-ray film to ensure the appropriate band had been excised. DNA was extracted from the piece of gel with 30 µl of water for 60 min at room temperature. The aqueous phase was taken immediately for reamplification, and the conditions of the first 20 cycles were as in the DD reaction. Another 20 cycles were added after primer and dNTP concentration were changed to 10 and 100 µM, respectively, and one more aliquot of the enzyme was added.

Cloning and Sequencing of Bands of Interest

Reamplified cDNA bands were recovered from the 4% agarose gels using the GETsorb kit (Genomed, Triangle Park, NC). Extracted DNA was further purified by Qiagick kit (Qiagen, Hilden, Germany) and cloned into the pGEM-T vector (Promega). Plasmid DNA was prepared using the Qiagen plasmid kit. Sequencing was carried out with the sequenase II kit from Amersham Pharmacia Biotech using pUCM13 forward or reverse sequencing primers. Analysis of sequences was carried out on the National Center for Biotechnology Information GenBank™ database (BLASTN algorithm) (25) or by searching the GenBank™ and EMBL data base with Fasta program (GGC Software, Madison, WI).

Primers (5'-3')

RP3 (DD antisense): 5′-GTTAGATGATAG-3′
P2 (DD sense): 5′-CGATGATGATG-3′

Northern Blot Analysis

Total RNA was prepared from DA-treated or untreated cells for Northern blot analysis. Each lane was loaded with 5–10 µg of RNA corresponding to 10⁶ cells. RNA was separated on 1% agarose, 2.2 M formaldehyde in 1× MOPS buffer (pH 7.0). Gels were blotted onto Whatman nylon membrane, and ultraviolet cross-linking was performed.

Probes were prepared by random priming using Rediprime kit (Amersham Pharmacia Biotech). Blots were washed at room temperature for short probes and at 65 °C for long probes with 0.1× SSC (1× SSC = 0.15 M NaCl and 0.015 M sodium citrate), 0.1% SDS and exposed to x-ray film. For RNA quantification, the intensity of bands was measured by both soft laser-scanning densitometer (Biomed Instruments Inc. Fullerton, CA) and 202D gel documentation system (Dinco and Rheumini, Jerusalem, Israel). As a control for gel loading and transfer, membranes were stripped and re-hybridized to an oligonucleotide of 18 S rRNA (see list of primers above), phosphorylated as described (26). RNA quantities in each lane were normalized against RNA level in each sample.

Polymerase Chain Reaction (PCR) Amplification

Alternative Reverse Transcription—DD—Reactions were carried out essentially as described by Liang and Pardee (15) and Bauer et al. (16). 50–100 ng of total RNA were used in each reverse transcription reaction in a volume of 20 µl. 3′ end primers were used at a concentration of 2.5 µM (see list of primers below), and dNTP concentration was 20 µM. Reactions were incubated at 42 °C for 90 min and at 95 °C for another 5 min and stored in aliquots at −70 °C. Control reactions were performed in the absence of enzyme. To validate the reproducibility of the altered expression, three different RNA preparations were rescreened by identical DD protocols.

Specific Reverse Transcription—With 0.5–1 µg of total RNA was primed with 1 µM sequence specific primer using conditions similar to those described for DD, with the exception that dNTP concentration was 1 mM, and no RNase inhibitor was added.

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sense. After packaging, the vector was inserted into Escherichia coli (XL1-Blue MRF'). The cDNA library contained 6 × 10^5 independent clones and was amplified once.

To screen the library, colonies were plated on 150-mm NZY agar plates (50,000 plaque-forming units/plate), duplicated on nitrocellulose filters, and lysed according to the manufacturer's instructions (Stratagene). The colonies were pre-hybridized for 2 h at 42 °C in a solution containing 2 × 1.4 PIPES buffer, 50% formamide, 0.5% SDS, and 100 μg/ml denatured sonicated salmon sperm DNA. The filters were then hybridized overnight at 42 °C in the same buffer containing 10^5 cpm/filter of 32P-labeled probe, washed at 65 °C in a solution containing 0.1× SSC buffer and 0.1% SDS, and exposed overnight at −80 °C to Fuji RX x-ray film.

**Cloning and cDNA Sequencing of the Chick Homologue of TCP-1**

Positive clones were converted to bacterial clones by excising the pKB-CMV from the ZAP Express vector using the "ExAssist" helper phage. DNA sequence of both strands of positive clones was analyzed by the chain termination method described previously by Sanger et al. (29). The sequence was determined by the Tel Aviv University Faculty of Life Sciences sequencing units (Applied Biosystems, Inc. DNA sequencer 377) beginning with T3 and T7 primers and then with inner primers according to the already known sequences.

**Preparation of Sympathetic Neuron Cytoplasm**

Western blot analysis was performed as described by Harlow and Lane (30) using 12.5% polyacrylamide gels (polyacrylamide gel electrophoresis). Each lane was loaded with an equal amount of cytoplasmic protein (30 μg), which, following electrophoresis, was transferred to an Immobilon polyvinylidiene disulfide membrane for 2 h. Blots were stained with Ponceau to verify that an equal amount of protein was loaded and blotted in each lane. Membranes were then probed with the antibodies as indicated at dilutions recommended by the supplier. The intensity of the signal was determined by ECL assay reagents.

**TCP-16 Antibody**

Polyclonal antibody raised against TCP-16 subunit was purchased from StressGene, Victoria, Canada.

**Oligonucleotide Treatments**—21-mer oligonucleotide targeted against TCP-16 mRNA was purchased from Microsynth, Balgach, Switzerland. Antisense, 5'-GTCCCGGCTTCTCCTGGCATCG-3', and sense, 5'-CGATGCGAGAACGCGGGAC-3', were phosphorothioated at the last three bases at the 3' end and fluorescently labeled with fluorescein at the 5' end. The oligonucleotides were dissolved (10 mM stock) in distilled water prior to use and diluted to concentrations of 0.5, 1, 2, 3, and 5 μM in serum-free medium, and treated for 17 h for their effect on TCP-16. Sympathetic neurons were prepared as described previously in density of 2.5 × 10^5 cells in 24-well plates. Neurons were treated with oligonucleotides 24 h after plating.

The role of TCP-16 in DA-induced apoptosis was assessed by examining whether its down-regulation can affect neuronal viability after DA exposure. Using fluorescently labeled oligonucleotide, we found that most of the neurons in the cultures absorbed the oligonucleotide. Neuronal viability was monitored at the end of the time period.

**Transfection of Cultured Sympathetic Neurons**—Chick embryo sympathetic neurons were prepared as previously by Zilkha-Falb et al. (8) and plated in density of 4 × 10^5 cells in 35-mm plates. Cells were transfected 24 h after plating in the presence of serum using FuGENE kit (Roche Molecular Biochemicals) according to the manufacturer's instructions. The neurons were co-transfected with green fluorescent protein (GFP) with the empty vector (pBK-CMV) or with TCP-16 gene (pBK-CMV-TCP-16). Transfections were carried out at a 1:10 ratio in favor of the TCP-16 plasmid.

**RESULTS**

**Isolation of DA-induced Apoptosis-related Genes (Fig. 1)—**

Cultured sympathetic neurons treated with 300 μM DA for 12 h showed cell viability to be −80% (8). This concentration of DA is within the estimated range (0.1–1 mM) of its normal levels in dopaminergic neurons (31–33). Subjecting treated and untreated neurons to DD yielded a large number of reproducible, differentially expressed genes that were either up- or down-regulated in response to DA treatment (Fig. 1A). As deduced from the sequence shown in Fig. 1B, the isolated gene is the chicken homologue of the mouse TCP-16 subunit protein. Since homology of this fragment to TCP-1 is 78% on the DNA level and 91% on the protein level, we refer to this molecule as part of the chicken heat shock protein, or chicken chaperonin.

**Cloning and Sequencing of the Chick TCP-16 Subunit (Figs. 2 and 3)—**

Sequence comparison of the chick TCP-16 subunit to other CCT subunits revealed a high degree of identity (30%). Computer analysis of the chick 6 subunit (Fig. 2) disclosed five conserved regions that are shared by other subunits of TCP-1. The GDGTT sequence in the third region was previously recognized by Lewis et al. (34) to be homologous to a nucleotide phosphate binding domain of cAMP-dependent kinase and other members of this family. Another motif shared by numerous ATP/GTP-binding proteins is (G/A)kGp(G/A)p(G/A)kGp(G/A)p(G/A)k, where k represents a purine and Gp represents a pyrimidine. The GDGTT sequence in the third region was previously recognized by Lewis et al. (34) to be homologous to a nucleotide phosphate binding domain of cAMP-dependent kinase and other members of this family. Another motif shared by numerous ATP/GTP-binding proteins is (G/A)XXXGK(T/S). This sequence motif, LIK1TGCTNPGTK, in the chick TCP-16 subunit (amino acids 370–382) is generally referred to as the A consensus sequence or the P loop (35, 36). It is thought to form a...
flexible loop between a β strand and an α helix that interacts with one phosphate group of the ATP/GTP. The sequence motif, RTSLGPKGMDKMI, which is found in the amino terminus of the chick TCP-1 protein (amino acid 46–58), is shared by other CCT-1 subunits (b, g, d, e, z, h, and u) and is referred to as the chaperonin TCP-1 signature. Comparison of the amino acid sequence of the chick d subunit to d subunits of other species (human, mouse, Caenorhabditis elegans, and yeast) revealed a high degree of conservation (Fig. 3). The alignment of the peptide sequences from the different species shows that the above motifs (motifs 1 to 5) are highly conserved. The cloned TCP-1 shows 94% similarity and 92% identity to the human TCP-1 on the protein level. In addition, comparison of the deduced amino acid sequence of our clone to HSP60 revealed that the above five motifs have homologous regions in the HSP60; the underlined amino acids in the legend to Fig. 2 are identical in the two proteins (TCP-1 and HSP60), whereas the adjacent amino acids are conserved with similar properties (polarity and charge). The two proteins share overall 30% homology, especially at the carboxyl terminus. Alignment of the TCP-1 subunit with HSP70 shows these proteins share no significant homology in the conserved regions.

Induction of TCP-1 Subunit mRNA during DA-triggered Neuronal Apoptosis (Figs. 1 and 4)—The DD experiment and the subsequent Northern blot analysis with the DD fragment indicated that the d subunit was up-regulated after 12 h of DA exposure (data not shown and Fig. 1). Exposure of sympathetic neurons to DA for different time periods revealed a single band of 1.9 kbp that showed elevated expression as a function of DA treatment (Fig. 4A). The level of TCP-1 transcript was elevated 3-fold after 1 h of DA treatment, 9-fold after 4 h, and fell to 7-fold after 6–12 h (Fig. 4B).

Induction of TCP-1 Protein Levels during DA-triggered Neuronal Apoptosis (Fig. 5)—The levels of TCP-1 subunit rose 3.6-fold after 1 h of DA treatment (Fig. 5, A and B), peaked at 7-fold from 4–6 h, and dropped to a 3-fold increase at 24 h. A
similar set of experiments (data not shown) using total cellular rather than cytoplasmic proteins yielded similar induction in TCP-1 levels, ruling out the possibility that DA causes changes in protein localization rather than up-regulation of TCP-1. A similar mode of induction in TCP-1 levels was obtained after NGF deprivation (Fig. 5C); the protein levels of TCP-1 increased 2-fold after 24 h of NGF deprivation and fell slightly to 1.6-fold increase after 48 h.

Reduction of TCP-1 Subunit Level by Specific Antisense Oligonucleotide (Fig. 6)—0.5 to 2 μM TCP-1 antisense did not reduce the TCP-1 protein level, whereas 3 and 5 μM significantly reduced its level (Fig. 6A). Identical concentrations of TCP-1 sense had no effect on TCP-1 levels. TCP-1 level was reduced during the first 24 h after the addition of the antisense and rose slightly at 40 h. The same experiment conducted with the sense sequence (Fig. 6B) had no effect on TCP-1 levels. DA treatment of the neurons after 14 h of exposure to antisense resulted in significantly reduced TCP-1 levels 12 and 16 h after that treatment compared with control levels (Fig. 6C). Similar treatment in the presence of the sense oligonucleotide had no effect on TCP-1 protein levels (Fig. 6C). These results suggest that antisense treatment down-regulates the expression levels of TCP-1 subunit in the presence of DA.

TCP-1 Down-regulation Inhibits DA-induced Apoptosis (Fig. 7)—Exposure of the neurons to antisense oligonucleotide for 14 h resulted in 82% neuronal viability compared with only 40% in the absence of the antisense (Fig. 7A). Similar treatment with sense oligonucleotide provided no protection against DA toxicity (Fig. 7A). Increasing antisense concentra-

![Fig. 3. Protein sequence alignment of chick TCP-1 homologue with other species.](image)

![Fig. 4. Up-regulation of TCP-16 mRNA during DA-induced apoptosis in sympathetic neurons. A, sympathetic neurons were exposed to DA for different durations ranging from 0.5 to 12 h. At each time point, total RNA (10 mg/lane) was prepared from untreated (time 0) and treated cells. The RNA was subjected to Northern blot analysis and hybridized with the full-length (1.9 kbp) TCP-16. The membrane was rehybridized with GAPDH. B, the levels of TCP-16 were quantified and normalized against GAPDH levels. Experiments were repeated twice, and the results are presented as % of control (untreated cells).](image)
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Fig. 5. Up-regulation of TCP-18 protein during DA-induced apoptosis and NGF deprivation in sympathetic neurons. A, sympathetic neurons were treated with DA for various time periods ranging from 1 to 24 h. Cells were harvested, and cytoplasmic proteins isolated from DA-treated and control untreated cells were separated on 12.5% polyacrylamide gel and blotted onto polyvinylidene disulfide membrane. The membrane was stained with Ponceau to verify that equal amounts of protein were transferred, after which the blot was reacted with anti-TCP-18 monoclonal antibody (1:3000). Secondary antibody was goat anti-mouse horseradish peroxidase-conjugated. The blots were developed using the ECL system, and a representative Western blot is shown. B, quantitative analysis of bands is represented as % of control untreated cells (n = 3). C, up-regulation of TCP-18 during NGF deprivation-induced apoptosis. Sympathetic neurons were deprived of NGF for 24 and 48 h and then treated as described in A.

DISCUSSION

Based on full-length sequence analysis, we propose that our newly cloned protein is the chick homologue of TCP-18. Several arguments support this notion. (a) Previous studies (22, 37–39) report that all CCT complex subunits have similar molecular masses, amino acid numbers, and isoelectric points. Our isolated clone also has these features: 536 amino acids, a predicted molecular mass of 57.7 kDa, and pI of 7.75. (b) Comparison of our isolated clone sequence to the computerized DNA and protein data bases revealed a high degree of homology to human TCP-18 (94% similarity and 92% on the protein level). (c) Sequence alignment by SeqVu Software, Sydney, Australia (Fig. 3) is consistent with previous studies that describe the HSP family as conserved through evolution (38, 39). (d) The isolated sequence contains the five conserved motifs (Fig. 2) that were already reported as conserved regions in all CCT subunits and in the mitochondrial chaperonin homologue HSP60. These motifs include the ATP binding domain and the ATPase activity domain (37, 39). (e) The newly cloned gene product could be immunoprecipitated by anti-TCP-18 (data not shown).

Contrary to studies (21, 41) claiming that the TCP-1 complex was not stress-induced, we demonstrated that TCP-18 was up-regulated both at the mRNA and protein levels during DA-induced apoptosis (Figs. 4, 5). Recent studies are consistent with our findings that CCT genes are stress-inducible. Kuo et al. (42) report the up-regulated mRNA levels of two CCT genes corresponding to the archaean Haloferax volcanii by heat and salt shocks. Lu and Ramos (43) report the down-regulation of a cDNA corresponding to the chaperonin containing TCP-1 after benz[a]pyrene treatment (which induces acquisition of highly proliferative phenotypes). Reperch et al. (44) show the differential up-regulation of cDNA corresponding to TCP-1 after apoptosis and tumor suppression. Interestingly, the sequence of the chick homologue of TCP-18 contains a putative heat shock consensus element (5'-NGAANNTTCN-3', where N is a nucleoside) at positions −77 to −70 responsible for the activation for eucaryal heat shock genes in the CCT promoter region (42).
thetic neurons were treated with antisense (light gray bars A) or sense oligonucleotide (dark gray bars) for 14 h, exposed to 300 \( \mu M \) DA for 18 h, and tested for neuronal viability. Under normal conditions, the rate of spontaneous apoptosis was around 1%. Since most of the neurons in cultures absorbed the oligonucleotides (sense or antisense), we could assay neuronal viability using trypan blue exclusion assay. Siter cultures treated with 300 \( \mu M \) DA in the absence of oligonucleotide served as controls (black bars). Each experiment was repeated three times. B, dose response of antisense protection from DA toxicity. Cultured sympathetic neurons were treated with various concentrations of antisense (0.1–5 \( \mu M \)) for 14 h, exposed to 300 \( \mu M \) DA for 18 h, and tested for neuronal viability. ***, \( p < 0.001 \) (between antisense and sense treatments). Error bars represent \( \pm \) S.E. Statistical analyses were performed with analysis of variance Fisher test.

The role of stress proteins in cell death and survival pathways is an intriguing and controversial issue. Freyaldenhoven and Ali (45) report that 1-methyl-4-phenylpyridium (MPP\(^+\)) treatment of cultured fibroblast was associated with induced level of HSP70 and that heat shock pretreatment of the cells before the addition of 1 mM MPP\(^+\) significantly attenuated cell death. Overexpression of HSP70 rat fibroblast cell line rendered them more resistant to MPP\(^+\) toxicity. Creagh and Cot-
