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TorsinA in PC12 Cells: Localization in the Endoplasmic Reticulum and Response to Stress

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Most cases of early-onset torsion dystonia are caused by deletion of GAG in the coding region of the DYT1 gene encoding torsinA. This autosomal dominant neurologic disorder is characterized by abnormal movements, believed to originate from neuronal dysfunction in the basal ganglia of the human brain. The torsins (torsinA and torsinB) are members of the "ATPases associated with a variety of cellular activities" (AAA^+) superfamily of proteins that mediate chaperone and other functions involved in conformational modeling of proteins, protection from stress, and targeting of proteins to cellular organelles. In this study, the intracellular localization and levels of endogenous torsin were evaluated in rat pheochromocytoma PC12 cells following differentiation and stress. TorsinA, apparent MW 37 kDa, cofractionates with markers for the microsomal/endoplasmic reticulum (ER) compartment and appears to reside primarily within the ER lumen based on protease resistance. TorsinA immunoreactivity colocalizes with the lumenal ER protein protein disulfide isomerase (PDI) and extends throughout neurites. Levels of torsinA did not increase notably in response to nerve growth factor-induced differentiation. None of the stress conditions tested, including heat shock and the unfolded protein response, affected torsinA, except for oxidative stress, which resulted in an increase in the apparent MW of torsinA and redistribution to protrusions from the cell surface. These findings are consistent with a relatively rapid covalent modification of torsinA in response to oxidative stress causing a change in state. Mutant torsinA may interfere with and/or compromise ER functions, especially in dopaminergic neurons, which have high levels of torsinA and are intrinsically vulnerable to oxidative stress.

Key words: dystonia; oxidative stress; endoplasmic reticulum; movement disorders; cell morphology

Dystonia is a neurologic condition associated with sustained muscle contractions and abnormal posturing of skeletal muscles throughout the body, which affects about 100,000 individuals in the United States (Fahn, 1988). Over 12 different genes have been implicated in various forms of hereditary dystonia, most causing an autosomal dominant condition with reduced penetrance (Fahn, 1988; Müller et al., 1998). The most severe form of hereditary dystonia, termed *early onset, generalized dystonia,* is caused by mutations in the TOR1A (DYT1) gene on chromosome 9q34 (Ozelius et al., 1997, 1998). Thirty to forty percent of heterozygous carriers of this mutation develop dystonic symptoms, typically between the ages of 9 and 13 years, with unaffected, mutant gene carriers over the age of 28 years effectively escaping the disease (Bressman et al., 2000). No notable neuropathologic changes have been found in the brains of these patients (Zeman and Dyken, 1967; Hedreen et al., 1988; Rostasy et al., 2002), although minor changes in dopamine metabolism and enlarged dopaminergic neurons have been observed (Furukawa et al., 2000; Augood et al., 2002; Rostasy et al., 2002). These findings suggest that dystonia may be caused by an imbalance in neuronal function in a critical window of postnatal development rather than degenerative death of neurons.

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Most cases of early-onset dystonia are caused by loss of a glutamic acid residue near the carboxy terminal end of torsinA (Ozelius et al., 1999; Klein et al., 1999). Only one other mutation has been found in this gene to date, an in-frame, 18-bp deletion, resulting in loss of six amino acids near the carboxy-terminal region of torsinA, which has been found in the heterozygous state in a case of early-onset dystonia with myoclonic and tic-like features (Leung et al., 2001). The message for torsinA is expressed in many different tissues (Ozelius et al., 1997); however, in the adult human brain, levels are highest in the dopaminergic neurons of the substantia nigra (Augood et al., 1998, 1999).

The DYT1 gene is a member of a gene family comprising three other highly homologous genes in the human genome (Breakefield et al., 2001). TOR1B, which is adjacent to DYT1 on chromosome 9q34, shares the same exon structure, has 70% homology in the coding region, and encodes torsinB (Ozelius et al., 1999). TOR2A (previously called *TORP1*) on chromosome 9 and TOR3A (TORP2) on chromosome 1, which share about 50% homology in the coding region with TOR1A, encode torp2A and torp3A, respectively (Ozelius et al., 1999). The torsins are predicted to share features of ATP-binding and/or ATPase activity, secondary configuration, and formation of oligomeric complexes with the AAA^+ superfamily of chaperone proteins (Neuwald et al., 1999). AAA^+ proteins serve a wide variety of cellular functions (Confalonieri and Duguet, 1995; Neuwald et al., 1999), including membrane and organelle trafficking (Vale, 2000) and protein renaturation/degradation critical to recovery from stress (Wickner et al., 1999).

In the present study, we took advantage of the relatively high levels of torsinA in the rat pheochromocytoma-derived cell line PC12 to evaluate the localization of the endogenous protein and its response to conditions of differentiation and stress. Differentiation was induced with nerve growth factor (NGF), which leads to assumption of many of the morphologic and biochemical properties of neurons, including neurite extension and the ability to synthesize dopamine (Greene and Rein, 1977; Rebois et al., 1980). The intracellular localization of torsinA was evaluated by subcellular fractionation and immunocytochemical analysis using antibodies to torsin and other marker proteins. PC12 cells were also exposed to a variety of stresses, including heat shock, ATP and/or glucose depletion, calcium ionophore, inhibition of protein glycosylation, ethanol, and oxidative stress. Levels and apparent size of torsin immunoreactivity were determined by Western blot analysis, and, for heat shock, the message level was evaluated by Northern blot analysis. Findings confirm a primary lumenal endoplasmic reticulum (ER) localization of endogenous torsinA with high sensitivity to oxidative stress.

MATERIALS AND METHODS

Cell Culture

Rat PC12 cells were obtained from several sources, ATCC, Dr. Arthur Tischler (New England Medical Center), and Dr. Thomas Martin (University of Wisconsin). Line PC12BAG was derived by stable transfection of the latter with a lacZ expression cassette (Short et al., 1990). (All PC12 cell strains used showed similar properties in these studies.) PC12 cells from ATCC and New England Medical Center were grown in Dulbecco's modified Eagle's medium (DMEM) with high glucose supplemented with 10% fetal calf serum (FCS), 10% horse serum, and 1% penicillin/streptomycin; those from the University of Wisconsin were grown in the same except with 5% calf serum, 5% horse serum, and no antibiotics. 293T/17 cells were obtained from Dr. David Baltimore (MIT) and grown in DMEM with 10% FCS and antibiotics. All cell culture reagents were obtained from Gibco (Grand Island, NY) or Hyclone (Logan, UT), unless otherwise noted. PC12 cells were differentiated by treatment with mouse NGF (mNGF 2.5S; Alomone Labs, Jerusalem, Israel) at 50 ng/ml for 4–5 days. Stress conditions included heat shock: exposure to 42°C for up to 3 hr with varying recovery periods; ATP depletion: treatment with 0.02% Na azide and 50 mM deoxyglucose in glucose-free, serum-free medium for 15 min (Nehls et al., 2000) or with 0.1 μM antimycin A in glucose-free, serum-free medium for 45 min (Pereira et al., 1998); ethanol: 50 mM ethanol for 12 hr (with no recovery); oxidative stress: 5 mM *tert*-butylhydroperoxide in serum-free medium for 40 min (Liu et al., 1998) or H_2O_2 at concentrations ranging from 1 μM to 12.5 mM for 1 hr in medium with or without serum and with and without recovery, as indicated; ER stress: treatment with 2 μM A23187, 1 μg/ml tunicamycin (Sigma, St. Louis, MO) for 16 hr or 1 μM geldanamycin (kindly provided by Dr. Len Neckers, NIH) for 16 hr; pathogen simulation: treatment with 2,000 U human α-interferon for 12 hr (no recovery); and neurotoxic insult: 100 μM kainic acid for 16 hr or 100 μM dopamine with 10 μM Cu^{2+} sulfate or Fe^{2+} citrate for 16 hr. Stressed cultures were given fresh growth medium after treatments of less than 16 hr (recovery period) and harvested together (unless otherwise indicated) with 16-hr-treated cultures by centrifugation for sodium dodecyl sulfate-polyacrlyamide gel electrophoresis (SDS-PAGE) analysis. Viability was determined by trypan blue exclusion or WST assay (Roche, Indianapolis, IN) for H_2O_2. For the latter, PC12 cells were plated at a density of 5×10^4 cells/well in a 96-well plate and 24 hr later incubated for 1 hr in serum-free medium without or with varying concentrations of H_2O_2, then rinsed and incubated with the WST reagent for 2 hr. Absorbance was read at 450 nm using a microplate spectrophotometer.

Subcellular Fractionation

Two confluent, 150-mm dishes of PC12 cells were lysed using a ball-bearing homogenizer (0.001 inch clearance) in 0.25 M sucrose with a protease inhibitor cocktail (PI; Boehringer Mannheim, Indianapolis, IN). Lysate was centrifuged for 5 min at 5,000 rpm in a microfuge to clear unlysed cells and nuclei. The supernatant from this spin (postnuclear supernatant) was loaded on top of 11 ml 0.25–1.9 M sucrose gradient containing 20 mM HEPES, pH 7.3, 1 mM EDTA, 1 mM EGTA, and PI. Gradients were centrifuged at 30,000 rpm in SW41 for 18 hr and then collected in 24 0.5-ml fractions. Samples were electrophoresed on standard 10–15% SDS-polyacrylamide gels, transferred to nitrocellulose using a Bio-

Rad (Hercules, CA) semidry transfer apparatus, and immunoblotted using the indicated antibodies. Blots were developed using horseradish perioxidase (HRP)-conjugated secondary antibodies and the ECL-Plus system (Amersham, Arlington Heights, IL) and a Molecular Dynamics (Sunnyvale, CA) Storm System 860 detecting blue fluorescence at a PMT voltage of 800.

Antibodies

Antibodies to torsin used in this study included rabbit antiserum TAB1 (affinity purified) to a synthetic peptide representing sequences (RVAEEMTFFPKEER) in the carboxy terminus of human torsinA; a mouse monoclonal antibody, D-MG10, generated against a GST-fusion protein bearing 42 amino acids within the carboxy terminal of torsinA corresponding to human exon 4 (both characterized by Hewett et al., 2000); and a mouse monoclonal antibody, D-M2A8, generated against an N-terminal fusion of maltose binding protein (MBP) to wild-type torsinA sequences lacking the 51 amino acids at the amino terminus that include the signal sequence and putative transmembrane domain, produced in bacteria (see recombinant MBP-torsinA below).

Other antibodies used included rabbit antisera: SPA-891 (1:1,500) to the ER protein, protein disulfide isomerase (PDI; Stressgen, Vancouver, British Columbia, Canada); SPA-860 (1:2,000) to the carboxy terminus of calnexin (Stressgen); anti-Hsp70 (HSP-72; 1:150), a cytoplasmic heat shock protein (Stressgen); and anti-Grp78 (1:1,000) to Grp78/BiP, an ER stress protein (Affinity Bioreagents Inc., Catalog No. PA1-014). Mouse monoclonal antibodies included β-COP (1:1,000) to the Golgi/intermediate compartment (Sigma; clone maD; Catalog No. G-6160), clone 3A10 (1:1,000) to P115 in the Golgi (provided by Dr. Gerry Waters, Princeton University), and C169.1 (1:1,000) to synaptobrevin (provided by Dr. Reinhard Jahn, MPI, Göttigen, Germany).

Purified Recombinant MBP-TorsinA

MBP-torsinA expression plasmid (pmal-C1-torA) and control MBP expression plasmid were used to transform *Escherichia coli* strain ER2508 (New England Biolabs, Beverly, MA), and, after induction with 0.5 mM IPTG (2 hr, 30°C), cells were lysed and the proteins bound to amylose resin (New England Biolabs) and eluted with maltose in buffer according to the manufacturer's instructions.

Immunoprecipitation

PC12 cells were harvested by trypsinization and centrifugation and washed twice in Ca^{2+}/Mg^{2+}-free PBS by repeated centrifugation (1,000*g* for 5 min) and resuspension. Pellets were resuspended in 1.2× pellet volume with lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% NP-40), incubated on ice for 30 min, and kept at 4°C throughout. Lysates were centrifugated at 17,000*g* for 30 min, and supernatants were precleared by incubation with normal rabbit serum (10 μl per 500 μl lysate) and protein A/G-agarose beads (1:1; 50 μl; Boehringer Mannheim, Indianapolis, IN) for 1 hr. After centrifugation, the supernatant was incubated with protein A/G-agarose and 10 μl TAB1 antiserum or preimmune overnight. Beads were collected by centrifugation, washed five times with 500 μl 1% NP-40 lysis buffer with PI by repeated centrifugation, and then resuspended in 60 μl 1× sample buffer for SDS-PAGE.

SDS-PAGE and Western Blots

Total cell lysates were prepared by washing the cells twice with PBS and resuspending the cell pellet in lysis buffer with protease inhibitors. Protein concentrations were determined using the Coomassie plus protein assay reagent (Pierce, Rockford, IL) and a bovine serum albumin (BSA) standard (Bio-Rad). Lysates (15–20 μg protein) were fractionated by electrophoresis in 12.5% polyacrylamide gels, unless otherwise indicated, according to the method of Laemmli (1970); electrophoretically transferred to nitrocellulose (Bio-Rad); and stained for total protein with 0.2% Ponceau-S (Sigma). After staining, the membranes were blocked overnight in 10% nonfat dry milk powder in 150 mM NaCl, 50 mM Tris, pH 7.9, 0.05% Tween (TBST). The blots were probed with torsin antibodies diluted in 2% milk in TBST, and the proteins were visualized with HRP-conjugated secondary antibodies and the ECL Reagent system (Amersham Pharmacia Biotech, Piscataway, NJ). Affinity-purified polyclonal TAB1 antibodies (Hewett et al., 2000) were used at a dilution of 1:40 or 1:60; the monoclonal antibody ascites fluids for D-MG10 or D-M2A8 were used directly (1:150 and 1:100, respectively) for Westerns and immunochemistry. Secondary antibodies for Western blots were sheep anti-mouse IgG-HRP (1:10,000; Amersham Pharmacia) and donkey anti-rabbit IgG-HRP (1:7,500; Amersham Pharmacia).

To evaluate posttranslation modifications, cell lysates (nondenatured) were treated with 1,500 U endoglycosidase H (endoH), 1,500 U peptide:N-glycosidase-F (PGNase F), or 40 U calf intestinal phosphatase (CIP; New England Biolabs) at 30°C for 16 hr prior to SDS-PAGE, according to the manufacturer's instructions (New England Biolabs). Carbonylation was evaluated using Oxybiot protein oxidation detection kit (Intergen, Purchase, NY) by derivatization of the carbonyl groups to 2,4-dinitrophenyl hydrazone (DNP), followed by Western blot analysis sequentially with DNP and torsin antibodies.

N-Terminal Sequencing of TorsinA

293T/17 cells (15 100-mm dishes) were transfected with the cDNA expression plasmid 2696 encoding the wild-type human torsinA cDNA (Hewett et al., 2000). Three days later, cells were harvested in lysis buffer with PI, and torsinA was immune precipitated with D-MG10 antibodies and protein A sepharose, as described above. Protein was resolved by SDS-PAGE on 10% acrylamine gel and blotted onto PVDF membranes (Bio-Rad); then, membrane was stained with amido black, and the 37 kDa band was excised and washed extensively in double-distilled H_2O. N-terminal sequencing was performed at the Harvard Microchemistry Laboratory (Cambridge, MA).

Protease Protection

Two confluent 150-mm dishes of PC12 cells were lysed as described above in 0.25 M sucrose, 10 mM HEPES, pH 7.4, 10 mM KCl, 1.5 mM $MgCl_2$, 5 mM EDTA, 5 mM EGTA. Postnuclear supernatant was centrifuged at 30,000 rpm in a TLA100.3 for 30 min. The pellet from this spin (microsomal pellet) was resuspended in lysis buffer containing 100 mM NaCl.

Sample was separated into four fractions. Two received Triton X-100 (1% final concentration). Proteinase K (Calbiochem, La Jolla, CA; 539480-2TU) was added (0.05 mg/ml final) to one sample with Triton and to one without Triton. Samples were incubated for 30 min on ice, and then the reaction was stopped by addition of PI + 5 mM phenylmethylsulfonyl fluoride (PMSF). Hot Laemmli SDS-sample buffer was then added, and samples wre immediately boiled for 5 min. Aliquots were subjected to immunoblot analysis with the indicated antibodies.

RNA Isolation and Northern Blot Analysis

Total RNA was isolated from cells with Trizol reagent (Gibco) according to manufacturer's instructions, electrophoresed on 0.66 M formaldehyde agarose gels, and transferred to nylon membranes (Hybond-XL; Amersham Pharmacia Biotech) by capillary transfer. After ultraviolet (UV) cross-linking (Stratalinker 2400; Stratagene, La Jolla, CA), nylon blots were hybridized with random primer ^{32}P-labeled cDNA probes (DECAprime II DNA labeling kit; Ambion, Austin, TX) overnight at 42°C according to standard procedures.

Templates for cDNA probes were generated by PCR from rat total brain cDNA on the basis of published sequences. For the 1,164-bp rat Hsp72 template (Genbank L16764; nucleotides 188–1,351), primer sequences F: 5′-AAACAGCGATCGGCATCGACC-3′ and R: 5′-GCAGGTCCTGCACGTTCTCCG-3′ were used at an annealing temperature of 63°C. The 753-bp rat cyclophilinB template was generated by nested polymerase chain reaction (PCR; annealing temperature 58°C) using the outer primer pair F: 5′-GCCTCTCGGAGCGCAATATG-3′ and R: 5′-AATCAGGCCTGTGGAATGTG-3′ and the inner primer pair F: 5′-TATGAAGGTGCTCTTCGCCG-3′ and R: 5′-CGTCGGATGGAAGCACTGGA-3′ (Genbank AF071225; nucleotides 22–774). A 980-bp rat torsinA probe was generated using primers F: 5′-CTGCTGGTGCCGTGCGTGGT-3′ and R: 5′-GGCAGCAGGGCTCAGTCATC-3′ (annealing temperature 61°C). This probe spans almost the entire rat cDNA extending 14 bp into the 3′ untranslated region (UTR). The PCR product was sequenced to verify the identity of rat torsinA message (Ziefer et al., 2002). Bands on autoradiographs were quantified on an LKB Ultrascan XL laser densitometer.

Immunocytochemistry

Cells grown on glass coverslips (coated with collagen; Biomedical Research) were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) at 37°C for 30 min. After thorough rinsing with PBS, cells were incubated with 0.1% NP-40 in PBS for 20 min, followed by blocking solution, 10% goat serum (Vector Laboratories, Burlingame, CA) in PBS for 1 hr. Cells were incubated with affinity-eluted monoclonal torsinA antibodies (1:100) with or without other antibodies (see above) for 1 hr. Rhodamine-conjugated goat anti-rabbit secondary antibody or fluorescein-conjugated goat anti-mouse secondary antibody (Tago Immunologicals, Camarillo, CA) was then used (30 min with 1:200 dilution in PBS plus 1% BSA). Coverslips were mounted onto slides using gelvatol mounting medium containing 15 μg/ml 1,4-diazabicyclo (2.2.2) octaine (Dabco; Aldrich Chemical Co., Milwaukee, WI), an antifade agent. Cells were examined on a Nikon fluorescence microscope using 40 × 1.3-N.A. and 60 × 1.4-N.A. objectives, with images recorded on Kodak Tri-X-Pan 400 (Eastman Kodak Co., Rochester, NY). Confocal images were generated with a Nikon TE 300 microscope and a Bio-Rad MRC 100 laser confocal imaging system. Controls included 1) preimmune sera and 2) no secondary antibody.

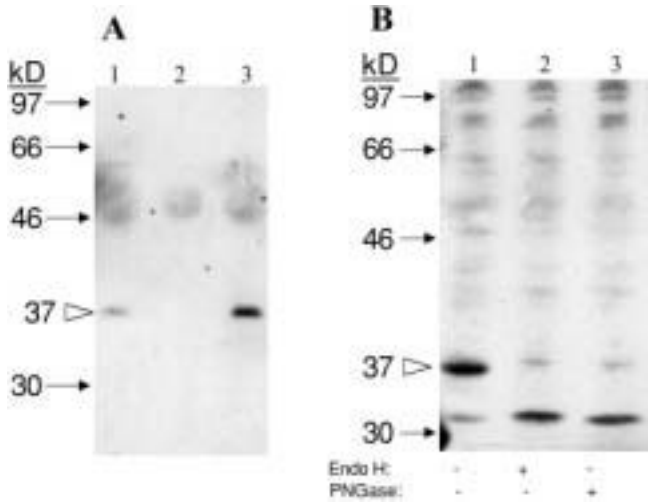

Fig. 1. Western blots of PC12 cell extracts following immune precipitation and deglycosylation. **A:** PC12 cell lysates were immune precipitated with TAB1 antibodies or preimmune serum and resolved by SDS-PAGE (12.5%); Western blot analysis was carried out with D-M2A8 antibodies. Lane 1: Total cell lysate (20 μg) without immune precipitation. Lane 2: Pellet from immune precipitation with preimmune serum from the same rabbit that later generated TAB1 antibodies. Lane 3: Pellet from immune precipitation with TAB1 antibodies. **B:** PC12 cell lysates (20 μg) were treated with endoH or PGNase F, and SDS-PAGE and Western blot analysis were carried out with D-MG10. Lane 1: Untreated lysate. Lane 2: Treated with endoH. Lane 3: Treated with PGNase F. Arrows, MW markers: 97 kDa, 66 kDa, 46 kDa, 30 kDa; arrowheads, 37-kDa torsinA.

RESULTS

Nature of Endogenous Torsin in PC12 Cells

Previous immunoprecipitation and Western blot analysis revealed that antibodies (polyclonal TAB1 and monoclonal D-MG10) prepared against sequences encoded in exon 4 and exon 5 of human TOR1A, respectively, recognized an immunoreactive band in PC12 cell lysates of approximately 37 kDa, the predicted size for torsinA, similar in size to the overexpressed protein (Hewett et al., 2000). Immunoprecipitation with TAB1 and Western blot analysis with monoclonal antibody D-M2A8 prepared against an MBP-torsinA fusion protein revealed that this latter antibody also detects the same 37-kDa protein in PC12 cell lysates (Fig. 1A). This immunoreactive band is referred to hereafter in this paper as torsinA. The amount of immunoreactive torsinA in PC12 cells was estimated as 2.5 μg/mg total cell protein by Western blot analysis in comparison with the dose-dependent intensity of immunostaining (TAB1) of a pu-

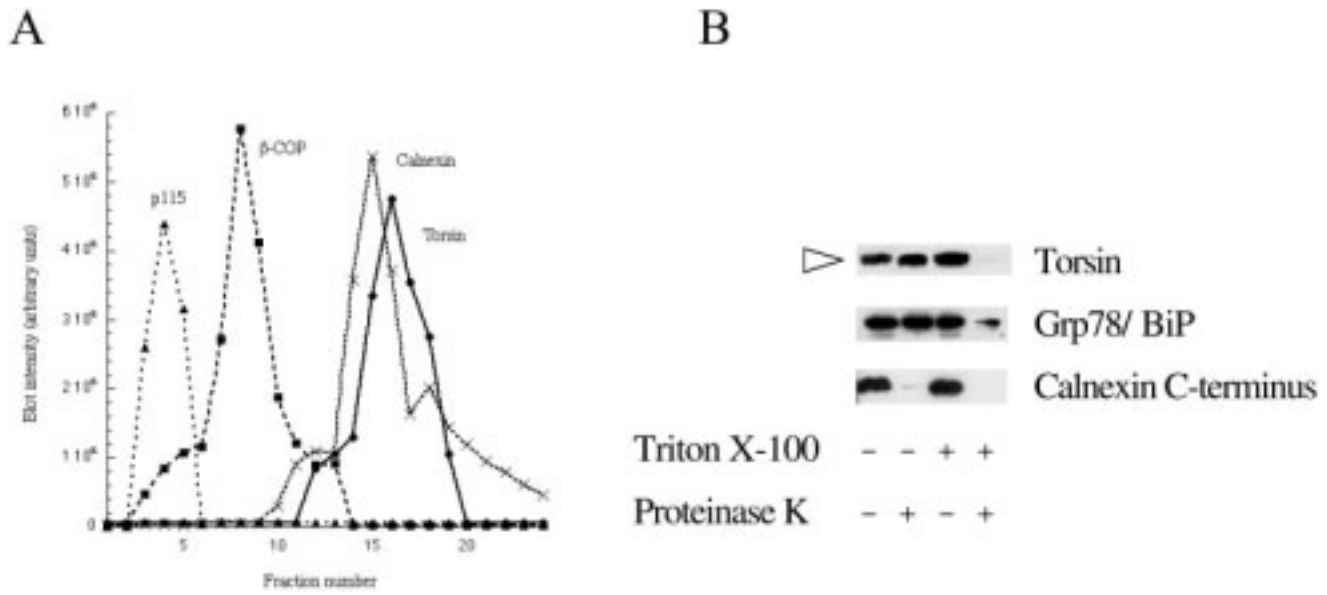

Fig. 2. Subcellular fractionation of torsin in PC12 cells and protease sensitivity. **A:** PC12 cells were lysed and membranes separated on a 0.25–1.9 M sucrose gradient (11 ml). Fractions (0.5 ml; lightest at the left, fraction 1) were collected and proteins resolved by SDS-PAGE and analyzed by immunoblot analysis for p115 (triangles, Golgi marker), β-COP (squares, Golgi and intermediate compartment), calnexin (crosses, ER), and torsin (circles, using TAB1 antibodies). Blots were quantitated using ECL-Plus reagents (Amersham) and a Molecular Dynamics Storm imaging system. **B:** Microsomal membranes from freshly lysed PC12 cells in buffer containing 100 mM NaCl were treated with proteinase K in the absence or presence of 1% Triton X-100. Samples were analyzed by SDS-PAGE and immunoblotting with antibodies against torsin (arrowhead; TAB1), Grp78/BiP, and the cytoplasmic tail of calnexin.

rified MBP-human torsinA fusion protein produced in *Escherichia coli*. TorsinA was reduced in apparent size to about 31 kDa by incubation of lysates with endoH, which deglycosylates high mannose and hybrid carbohydrates characteristic of ER resident proteins, as well as with PGNase F, which cleaves these as well as complex oligosaccharides modified in the Golgi and subsequent cellular compartments (Fig. 1B). This high-mannose glycosylation pattern of torsinA is consistent with primary residence in the ER.

Quantitative immunoblots following fractionation of PC12 cell homogenates by sucrose density gradient centrifugation confirmed that torsinA resides in the microsomal fraction with the ER proteins calnexin (Fig. 2A) and Grp78/BiP (not shown) and not with marker proteins for membranes from compartments farther along the secretory pathway, including the Golgi/intermediate protein β-COP and the Golgi marker p115. To test the orientation of torsinA with respect to the ER membrane, a microsomal membrane fraction of PC12 cells was treated with proteinase K with or without addition of detergent (Fig. 2B). In the absence of Triton X-100, both a known lumenal protein Grp78/BiP and torsinA were resistant to proteinase K digestion, whereas the cytoplasmic tail of the ER protein calnexin was digested. In the presence of Triton X-100, all three proteins were digested. Resistance to protease digestion in the absence of detergent confirms that the bulk of endogenous torsinA, as with Grp78/BiP, resides within the lumen of a membrane compartment, most likely the ER. Entry into the ER was previously predicted by a putative signal sequence at the amino terminus of torsinA (Ozelius et al., 1997). Cleavage at amino acid 20, in the N-terminal sequence mklgravlgllllapsvvqa/vepislglalagvltgyiy, during translation was verified by transfection of 293 cells with a full-length torsinA cDNA expression cassette, followed by immune precipitation, gel isolation, and N-terminal sequencing of the overexpressed protein. The cleaved N-terminal region of 19 hydrophobic amino acids is predicted to be a transmembrane domain (Ozelius et al., 1997).

Response of Torsin to Differentiation and Stress

Western blot analysis was used to assess the size and relative amounts of the 37-kDa torsin protein under different culture conditions. Although in some experiments there appeared to be an increase in the apparent amount of torsinA in PC12 cells after 4 days of NGF treatment, as cells developed neurite-like processes, this was not consistent across experiments, and longer treatment times did not sustain an increase. Moreover, levels of the torsin message did not increase with NGF incubation for 12, 24, 48, or 72 hr, as assessed by Northern blot analysis (data not shown). Given torsin's homology with heat shock proteins, e.g., hsp104 (Ozelius et al., 1997), heat inducibility was assessed both at the message and at the protein levels. Northern blot analysis of the torsinA message and a constitutive message for cyclophilinB showed that both remained unchanged in response to heat shock, whereas the

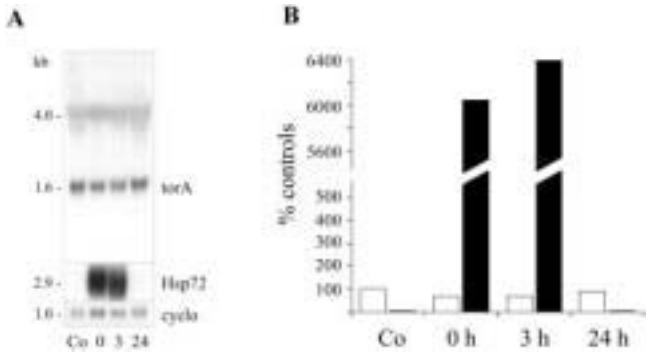

Fig. 3. TorsinA and Hsp72 mRNA levels in heat-shocked PC12 cells. **A:** Northern blot of total RNA from PC12 cells without treatment (Co) and 0, 3, and 24 hr after a 3-hr heat shock at 42°C hybridized consecutively to ^{32}P-labeled probes for rat torsinA (torA), Hsp72, and cyclophilinB (cyclo). Molecular weights are indicated in kilobases. The faint, broad bands at just about 4.0 kb in the blot hybridized with the torsinA probe are identical in size and shape to the 28S ribosomal RNA band on the ethidium bromide-stained gel (not shown) and represent a cross-hybridization. **B:** Bar diagram of the Northern blot results after densitometric quantification, showing relative amounts of torsinA (open bars) and Hsp72 (solid bars) hybridization signals normalized to the cyclophilinB message.

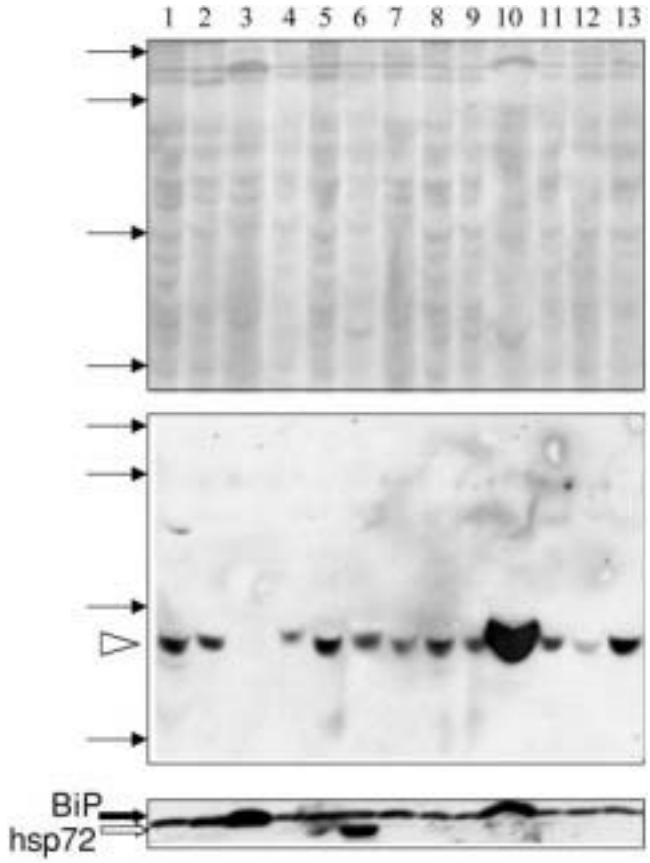

Fig. 4. Western blot showing PC12 cells exposed to various stresses. Cells were exposed to a variety of stress conditions, then harvested 16 hr later, and cell lysates (20 μg) were resolved by 12.5% SDS-PAGE and immunoblotted using torsinA antibody D-M2A8 or antibodies for stress-responsive proteins BiP and hsp72. Cells were treated as follows: lanes 1, untreated cells; 2, geldanamycin; 3, tunicamycin; 4, antimycin A; 5, kainic acid; 6, heat shock; 7, α-interferon; 8, Ca^{2+} ionophore; 9, ATP depletion; 10, *tert*-butylhydroxyperoxide; 11, dopamine and Fe^{2+}; 12, ethanol; 13, dopamine and Cu^{2+} (see Materials and Methods for concentrations and length of exposure). **Top:** Ponceau staining of protein. **Middle:** D-M2A8 immunoblot (arrowhead, torsinA); arrows indicate same MW markers as in Figure 1. **Bottom:** Portion of same blot stripped and immunostained for BiP/GRP78 (solid arrow) and hsp72 (open arrow).

levels of the hsp72 message were found to increase dramatically after this treatment (Fig. 3). Amounts of hsp72 protein also increased in response to heat, but there was no notable increase in levels of torsinA on Western blot analysis (see below).

In addition to heat shock, there are a number of other stresses with which cells must contend. These include ER stresses caused by protein overload and accumulation of unfolded, unprocessed proteins; influx of calcium ions; free radicals; oxidative stress; toxins; and pathogens (Kaufman, 1999). Levels and size of torsinA in PC12 cells were evaluated by Western blot analysis after a variety of sublethal stresses, including ER stresses, such as geldanamycin, which inhibits the cytoplasmic chaperone protein hsp 90 (Neckers, 2000); tunicamycin, which blocks glycosylation (Kaufman, 1999); and A23187, which increases Ca^{2+} in cells; and oxidative stresses, such as antimycin A in glucose-free medium to block oxidative phosphorylation (Pereira et al., 1998); sodium azide and deoxyglucose to deplete ATP (Nehls et al., 2000); *tert*-butylhydroperoxide (Liu et al., 1998) to increase H_2O_2 levels; and dopamine with heavy metals; and exposure to toxic compounds, such as kainic acid or ethanol (Chen and Sulik, 1996); or to α-interferon (Caraglia et al., 1999). Cells were harvested after 16 hr, and viability at that time was determined by trypan blue exclusion to be between 60% and 100% across samples. Only one of these stresses, *tert*-butylhydroperoxide, caused marked changes in immunoreactive torsin, consisting of an increase in both apparent amount and MW of the 37-kDa torsinA-immunoreactive band (Fig. 4). (Note that this increase in apparent amount was not reproducible with all torsin antibodies and appears to reflect increased affinity of some antibodies to oxidized torsinA.) Anticipated increases in amounts of Grp78/BiP, an ER stress marker, were seen in response to both tunicamycin and *tert*-butylhydroperoxide and of hsp72, a heat shock protein, in response to 42°C.

To determine the significance of changes in torsinA in response to oxidative stress, a dose response analysis to H_2O_2 was carried out in serum-free medium. Cells were exposed to 5–100 μM H_2O_2 for 1 hr and then harvested for Western blot analysis (Fig. 5A). With D-M2A8 (as well as other) antibodies, an apparent increase in MW of about 200 Da was noted at 5–100 μM H_2O_2, as resolved by using 16% polyacrylamide gels (Fig. 5B). The H_2O_2-induced changes in apparent MW did not appear to be due

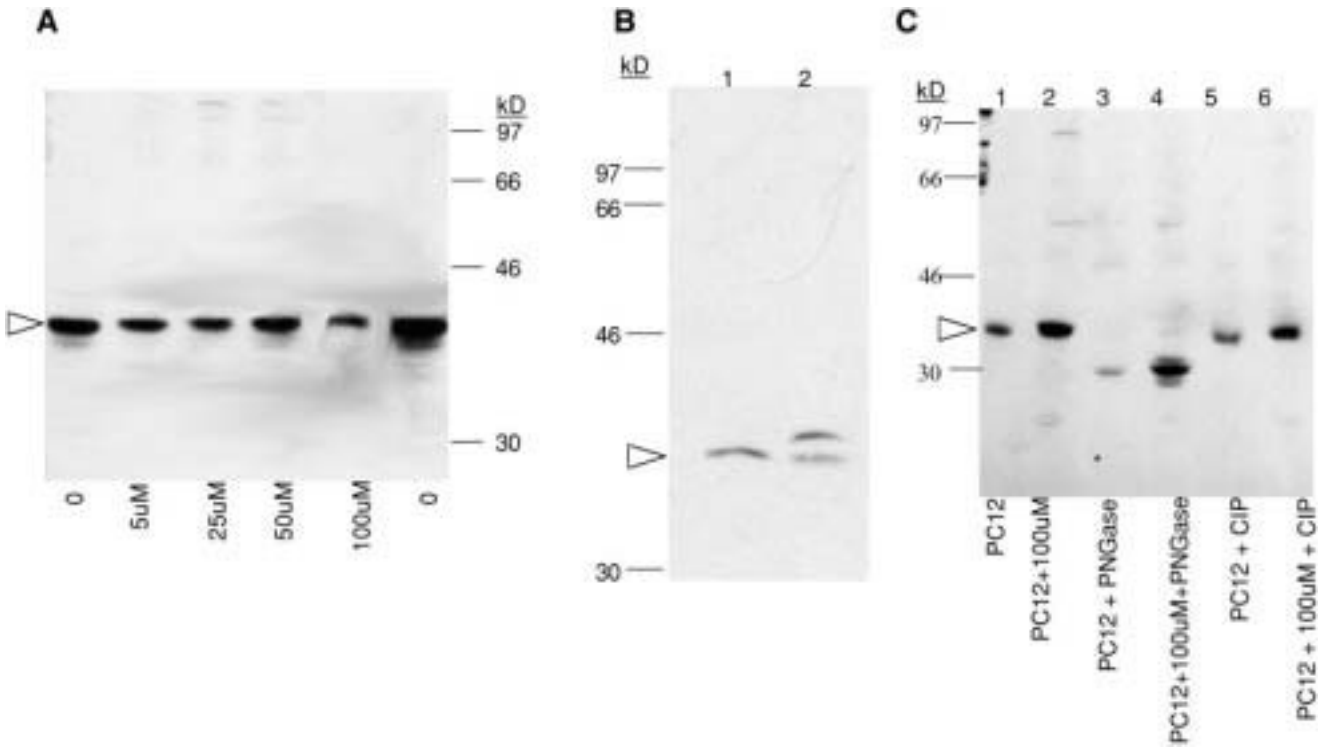

Fig. 5. Western blots showing response of torsinA to 5–100 μM H_2O_2. **A:** PC12 cells were exposed to 0, 5, 25, 50, or 100 μM for 1 hr in serum-free medium and harvested immediately thereafter, and lysates (20 μg) were resolved by SDS-PAGE (12.5%) and immunoblotted for torsinA. **B:** Lysates from untreated PC12 cells (lane 1, 20 μg) and untreated PC12 cells mixed 1:1 with PC12 cells treated with 100 μM H_2O_2 for 1 hr (lane 2; 20 μg each) were resolved in a 16% polyacrylamide gel and immunoblotted for torsinA. **C:** Cells were exposed to 0 μM (lanes 1, 3, 5) or 100 μM (lanes 2, 4, 6) H_2O_2 for 1 hr. Lysates were untreated (lanes 1, 2) or treated with PGNase (lanes 3, 4) or CIP (lanes 5, 6), and proteins resolved by SDS-PAGE and immunoblotting. All blots were probed with antibody D-M2A8; arrowheads, 37-kDa torsin.

to glycosylation or phosphorylation (Fig. 4C), in that, after PGNase treatment, immunoreactive bands decreased proportionally for both H_2O_2 and untreated cells, with no change in size apparent after CIP treatment. WST assays were determined in parallel experiments, with cells undergoing a dose-dependent loss in viability following 1 hr of exposure to H_2O_2, with no significant loss up to 50 μM and a 50% loss at about 100 μM (data not shown).

Dynamics of Torsin Localization in PC12 Cells

The intracellular distribution of immunoreactive endogenous torsinA was evaluated by immunocytochemistry in NGF-differentiated PC12 cells. TorsinA immunoreactivity was found throughout the cytoplasm, excluding the nucleus proper, and extending throughout the processes (Fig. 6A). Extensive colocalization was also seen with the ER marker PDI and torsinA, with somewhat more torsinA in the perinuclear regions compared with PDI (Fig. 6A–C).

A possible physiologic significance of the response to H_2O_2 is suggested by changes in the subcellular distribution of torsinA in response to exposure to H_2O_2. Exposure to 5 μM H_2O_2 for 1 hr resulted in a redistribution of some torsinA immunoreactivity to cell surface protrusions, "blebs" (Fig. 6D–F). Interestingly, these protrusions, which stain intensely for torsinA, were virtually unstained with antibodies to the ER marker PDI. The distribution of PDI did not change notably from that in untreated cells, although it appeared more clumped (cf. Fig. 6B and E). The change in the subcellular distribution of torsinA at increasing concentrations of H_2O_2 for 1 hr is striking, with most cells showing cytoplasmic staining with concentration around the nucleus at 1 μM (Fig. 6G), increased staining in the cell periphery and process endings at 3 μM (Fig. 6H), and marked intensity in bleb-like projections from the cell surface at 5 μM (Fig. 6I).

DISCUSSION

Endogenous torsinA in PC12 cells appears to reside primarily in the ER lumen. This localization is supported by high mannose glycosylation, enrichment with the ER lumenal marker calnexin in subfractions of cellular membranes, resistance to protease in membrane fractions in the absence of detergent, and cleavage of the predicted N-terminal signal sequence. PC12 cells normally have concentrations of immunoreactive torsinA in the range of 2.5 μg/mg total cell protein localized throughout the cell body and processes. TorsinA does not appear to be a

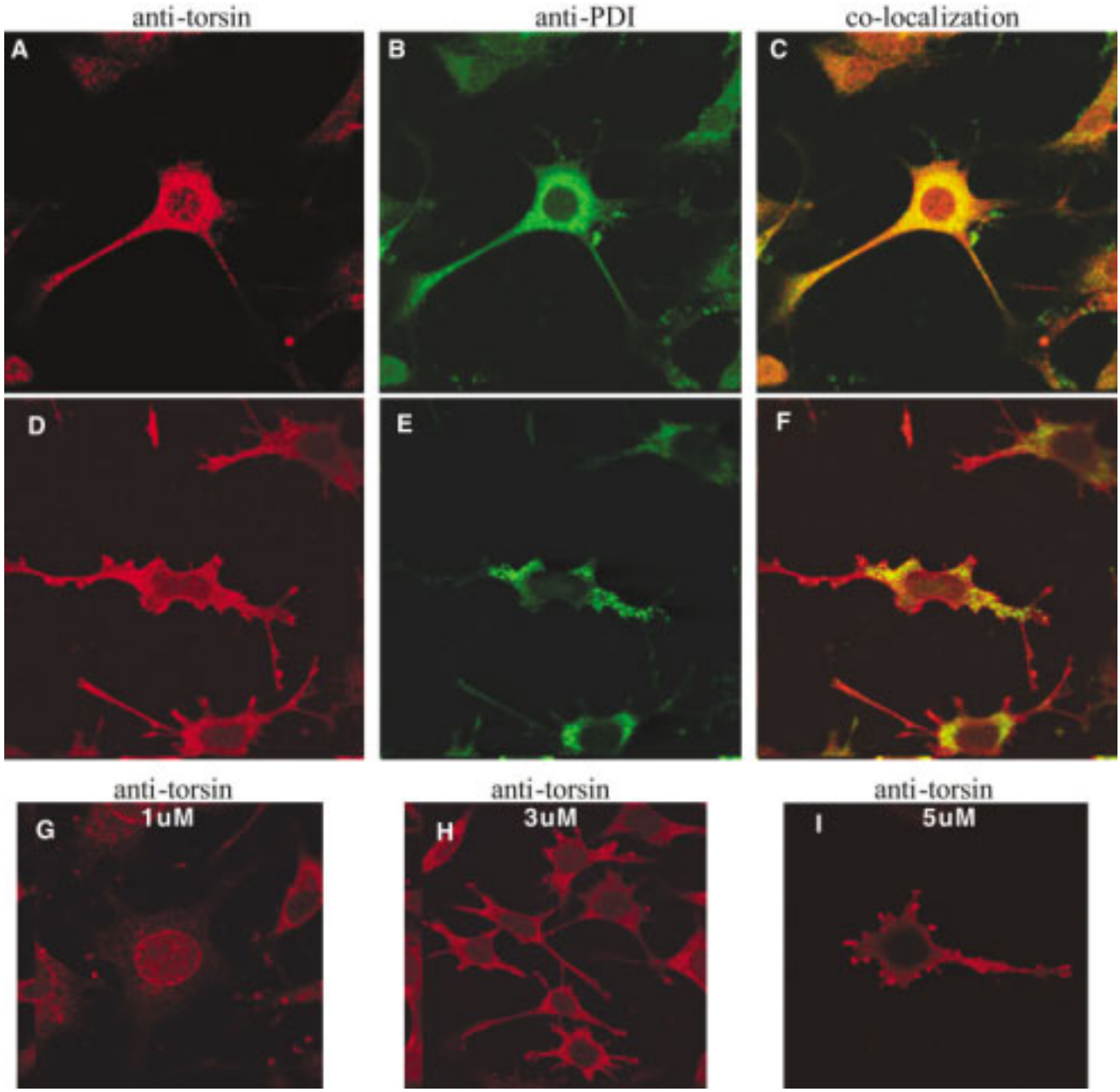

Fig. 6. Immunocytochemistry of torsinA and PDI in PC12 cells under various conditions. **A–C:** Control cells in growth medium. **D–F:** Cells exposed to 5 μM H_2O_2 for 1 hr in serum-free medium. In D–F, cells were costained for torsinA (D-MG10) and PDI, with merged images (colocalization). In **G–I**, cells were exposed to increasing concentrations of H_2O_2 for 1 hr in serum-free medium (G: 1 μM; H: 3 μM; I: 5 μM) and stained with D-M2A8 antibodies to torsinA. ×100.

classical "heat shock" type of chaperone protein, insofar as it is not up-regulated in response to a variety of stresses, including many ER stresses, such as the unfolded protein response, ATP depletion, heat, ethanol, α-interferon, or kainic acid. However, torsinA appears to be sensitive to some types of oxidation stress. At micromolar concentrations of H_2O_2, immunoreactivity shifted from a codistribution throughout the cytoplasm with the ER marker PDI to an increased intensity in the perinuclear region (1 μM for 1 hr) and then into protrusions from the cell surface (5 μM for 1 hr). This change in position was associated with an increase in the apparent size of torsinA from 37 kDa to about 37.2 kDa, suggesting a relatively rapid covalent modification of torsinA in response to

oxidative stress affecting its position in the cell. The biochemical nature of the changes in apparent size and intensity of torsinA observed in response to H_2O_2 is not known but does not appear to involve additional glycosylation, carbonylation, or phosphorylation.

In addition to processing of many proteins, the ER serves as the primary site of intracellular membrane synthesis from which the nuclear membrane and various vesicular compartments are derived (Kaufman, 1999). Although the torsins lack an apparent ER retention signal (Ozelius et al., 1997), they contain a hydrophobic domain (19 amino acids) downstream of the signal sequence at the N-terminus (Ozelius et al., 1997), which may serve to anchor them in the ER membrane. Given the partial disassociation between immunocytochemical staining for torsinA and the ER marker PDI, at low H_2O_2 concentrations, it is also possible that a subfraction of torsinA leaves the ER compartment proper or participates in a restructuring of a subcomponent of ER under some conditions. Initial localization studies, following transient transfection of cultured cells with wild-type torsinA constructs yielding overexpression of this protein, are consistent with a primarily ER/vesicular location, whereas overexpression of the "GAG-deleted" mutant protein generated large, spheroid inclusions (Hewett et al., 2000; Kustedjo et al., 2000). These inclusions consisted of whorled membranes at the ultrastructural level, which were positive for both torsin and PDI (Hewett et al., 2000). Other studies determined that these inclusions are not positive, however, for the ER marker BiP/Grp78 (Kustedjo et al., 2000), so their composition may also represent a subcomponent of the ER. A role for torsins in ER membrane orientation is supported by studies of a family member, OOC-5, in nematodes, which is critical to rotation of the nucleus relative to cortical patches on the cell surface during early embryogenesis (Basham and Rose, 1999, 2001).

The membership of the torsins in the AAA^+ family of chaperone proteins suggests a possible role in response to stress, with the ER being a very stress-sensitive compartment (Kaufman, 1999). Several types of stress responses have been observed in eukaryotic cells, including a cytoplasmic response triggered by stresses such as heat, heavy metals, and UV, typified by induction of hsp70 (hsp72; Morimoto, 1991); an ER response triggered by low glucose, excess unfolded proteins in the ER, and loss of calcium homeostasis, characterized by increases in ER chaperones such as BiP/Grp78 and Grp94 (Kozutsumi et al., 1988; Lee, 1992; Kaufman, 1999); and an ER/mitochondrial response to inhibition of oxidative phosphorylation and free radicals, characterized by modification of the iron response protein (IRP-1; Pantopoulos and Hentze, 1998). In the present study, after screening of a wide variety of stresses, immunoreactive torsinA was found to respond dramatically to oxidative stress caused by H_2O_2 with an increase in apparent MW and a partial redistribution of immunoreactive torsinA from the cytoplasmic space to membrane protrusions. The rapid time course (1 hr) during which the increase in MW takes place suggests that it is not due to new protein synthesis but rather to a covalent modification of torsinA. The low H_2O_2 concentrations (1–5 μM) at which these changes take place suggest they are physiologically relevant. Examples of other oxidation sensitive proteins include the hypoxia-inducible transcription factor (HIP) that is regulated by hydroxylation of a proline residue in response to oxidative conditions (Jaakkola et al., 2001; Yu et al., 2001). Oxidative stress is known to induce synthesis of other ER proteins, such as BiP/Grp78 and calreticulin (Liu et al., 1997), that serve to protect cells from toxic consequences (Liu et al., 1998). ER stress responses have been invoked in a number of hereditary human diseases, such as cystic fibrosis, spinocerebellar ataxia (SCA), Alzheimer's disease, and prion diseases, which are typically characterized by accumulation of protein aggregates believed to disrupt cell function and lead to cell death (Thomas et al., 1995; Aridor and Balch, 1999; Ron, 2002). Torsin appears to be distinct in this regard; although overexpression of mutant torsinA can lead to whorled membrane inclusions in cultured cells (Hewett et al., 2000; Kustedjo et al., 2000), there is no evidence of neuronal degeneration or presence of torsin-immunoreactive inclusions in brains from GAG-deleted dystonia patients (Hedreen et al., 1988; Rostasy et al., 2002; Walker et al., 2002). Interestingly, however, formation of α-synuclein inclusions in cultured cells can be blocked by overexpression of wild-type torsinA, supporting a role in protein degradation (McLean et al., 2002).

TorsinA is one of the first mammalian AAA^+ ER chaperone proteins identified to date. It is responsive to oxidative stress and may be involved in protein processing or degradation, vectorial movement of ER-derived membranes, and/or stress signaling (Breakefield et al., 2001; Basham and Rose, 2001; McLean et al., 2002). These aspects of torsinA are not mutually exclusive; the ER is actively involved in secretion of proteins, serves as the site of lipid and membrane synthesis (and, as such, determines the shape and fate of membranes), and is highly sensitive to oxidatively induced chemical changes (Sayre et al., 1999). All these functions are particularly critical to neurons, in that their long, complex projections require continuous, vectorial transport of membranes and proteins through dendrites and axons to maintain and regulate a variety of precise synaptic connections. TorsinA, by analogy, may be involved in tubular-vesicular membrane trafficking in neuronal processes (Nakata et al., 1988; Feiguin et al., 1994; Ahmari et al., 2000), as supported by the concentration of immunoreactivity in neuronal processes and at synaptic terminals in human and monkey brain (Augood et al., submitted). Furthermore, the brain is especially sensitive to oxidative damage produced by free hydroxyl radicals (generated from H_2O_2 and Fe^{2+}) by virtue of its low levels of antioxidants and high levels of O_2 consumption and free iron, as well as the presence of oxidizable substrates, such as catecholamines and unsaturated lipids (Nappi and Vass, 2000). Expression of mutant torsinA may

serve to reduce activity of the normal protein, as predicted from an oligomeric structure of the active form (Breakefield et al., 2001), such that, in mutant gene carriers, torsinA function is compromised, especially under conditions of oxidative stress or peak periods of synaptic plasticity during motor learning.

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