Early and Late Events Induced by PolyQ-expanded Proteins

IDENTIFICATION OF A COMMON PATHOGENIC PROPERTY OF POLYQ-EXPANDED PROTEINS*‡

To find a common pathogenetic trait induced by polyQ-expanded proteins, we have used a conditional expression system in PC12 cells to tune the expression of these proteins and analyze the early and late consequences of their expression. We find that expression for 3 h of a polyQ-expanded protein stimulates cellular reactive oxygen species (ROS) levels and significantly reduces the mitochondrial electrochemical gradient. 24–36 h later, ROS induce DNA damage and activation of the checkpoint kinase, ATM. DNA damage signatures are reversible and persist as long as polyQ-expanded proteins are expressed. Transcription of neural and stress response genes is down-regulated in these cells. Selective inhibition of ATM or histone deacetylase rescues transcription and restores the expression of silenced genes. Eventually, after 1 week, the expression of polyQ-expanded protein also induces endoplasmic reticulum stress. As to the primary mechanism responsible for ROS generation, we find that polyQ-expanded proteins, including native Ataxin-2 and Huntingtin, are selectively sequestered in the lipid raft membrane compartment and interact with gp91, the membrane NADPH-oxidase subunit. Selective inhibition of NADPH oxidase or silencing of H-Ras signaling with Metyrapone, the lipid raft activator of the resident NADPH oxidase, triggers a signal linking H-Ras, ROS, and ERK1/2 that maintains and propagates the ROS wave to the nucleus. This mechanism may represent the common pathogenetic signature of all polyQ-expanded proteins independently of the specific context or the function of the native wild type protein.

Polyglutamine diseases are caused by the expansion of the trinucleotide repeat (CAG) that is translated into an abnormally long polyglutamine tract (polyQ) in the native proteins. There are 10 neurodegenerative hereditary diseases caused by amplification of the CAG repeat in the coding region of specific genes; Huntington disease (HD), spinobulbar atrophy, dento-pallido-rubro-luysian ataxia, and seven spinocerebellar ataxias (SCA1, -2, -3, SCA-6 -7, SCA-12 -17). In most cases the physiological function of the expanded proteins is unknown (1).

The pathogenic mechanisms underlying such diseases seem to derive from the common signature present in all involved genes, i.e. amplification of a CAG repeat encoding the amino acid glutamine (Q) in the context of different proteins. Although the expanded proteins found so far are expressed in all tissues, the death induced by these proteins affects only specific neurons. The hallmark of these diseases is the accumulation of nuclear aggregates containing fragments of polyQ proteins (2–4).

We have developed a system of neuronal cells (PC12) that recapitulates the phenotypes induced by CAG expanded proteins in vivo. Cells expressing a synthetic fusion protein (HA-43Q-GFP) accumulate nuclear aggregates, inhibit cAMP- and NGF-dependent transcription, and slow down differentiation. These processes are modulated by conditional expression of polyQ proteins (5). This system, at variance with the animal models, allows 1) a precise temporal analysis of the effects induced by polyQ-expanded proteins and 2) the analysis of reversibility of the phenotypes induced by the polyQ proteins.

By switching on and off the expression of expanded polyQ proteins, we have monitored the early events induced by these proteins in different cell compartments: mitochondria, ER, and nucleus. We have found that polyQ-expanded proteins induce ROS and dissipation of the mitochondrial electrochemical gradient very rapidly (3 h). This is followed by a significant DNA damage response; ATM is activated in a time- and dose-dependent fashion and inhibits transcription. These events are selectively induced by the 43Q-repeat-containing

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3 The abbreviations used are: HD, Huntington disease; SCA, spinocerebellar ataxias; ER, endoplasmic reticulum; AEBSF, 4-(2-aminooethyl) benzenesulfonyl fluoride; TMRE, tetramethylrhodamine ethyl ester; Dox, doxycycline; NAC, N-acetylcysteine; 8-oxodG, 8-Oxo-7,8-dihydro-2’-deoxyguanosine; ROS, reactive oxygen species; HDAC, histone deacetylase.
Common Pathogenic Trait of PolyQ-expanded Proteins

protein and are present in primary fibroblasts of SCA-2 and HD patients.

We find that a distinctive feature of the polyQ-expanded proteins, including expanded Huntingtin and Ataxin-2, is the segregation in the lipid raft membrane compartment. In this membrane domain polyQ-expanded proteins interact with NADPH oxidase membrane subunit, gp91, and facilitate the activation of the enzyme. This event appears to be primarily responsible for the generation, diffusion, and maintenance of the oxidative wave that induces mitochondrial distress, DNA damage, and inhibition of neural-specific transcription.

EXPERIMENTAL PROCEDURES

Reagents—Dulbecco’s modified Eagle’s medium, FCS, L-glutamine, and pen-strept-anfotB solution were obtained from Invitrogen. 4-(2-Aminoethyl) benzenesulfonyl fluoride (AEBSF), apocynin, and diphenyleneiodonium chloride were from Sigma. Chimeric peptides (ggp91-TAT and control peptide TAT) were synthesized as described in Rey et al. (6) and used at a concentration of 10 μM.

The following primary antibodies were used: anti-HA (Roche Applied Science), anti-α-actinin (Sigma), anti-p(Ser139)-H2AX (Upstate Biotechnology), anti-p-(Ser/Thr) ATM/ATR substrate mouse monoclonal (Cell Signaling Technology), anti-H2A, anti-p-ERK, mouse monoclonal, anti-ERK1/2, goat polyclonal, anti-H-Ras mouse monoclonal, anti-gp91 phox goat monoclonal; anti-ATX-2 mouse monoclonal (Santa Cruz Biotechnology). Anti-mouse IgG, anti-rabbit IgG, and anti-goat IgG coupled to peroxidase were used as secondary antibodies (Sigma). Chemiluminescent (ECL) and fluorescent signals were quantified by scanning densitometry using ImageJ 1.43d software (Wayne Rasband, NIH (rsb.info.nih.gov). The antibodies were used according to the protocol described by the supplier.

Cell Culture and Transfections—All reagents for cell culture and transfection, unless otherwise indicated, were provided by Invitrogen. Tet-Off PC12 expressing HA-17Q-GFP or HA-43Q-GFP were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated horse serum, 5% fetal bovine serum, 1 mM glutamine, 100 mg/ml streptomycin, 100 units/ml penicillin, 100 mg/ml G418, 100 μg/ml hygromycin, and 10 ng/ml doxycycline hydrochloride in a humidified 5% CO2 atmosphere at 37 °C as described previously (5).

Human neuroblastoma SK-N-BE cells (American Type Culture Collection, LGC-Promochem Teddington, Middlesex, UK) were maintained in a mixture of RPMI 1640 medium containing 10% fetal bovine serum, 100 μg/ml glutamine, 100 μg/ml streptomycin, and 100 units/ml penicillin in 5% CO2 at 37 °C. Cells were transfected using Effectene according to the manufacturer’s instructions (Effectene transfection reagent, Qiagen) at 40–60% confluence with 2.5 μg of expression vectors coding for a fusion protein containing at the NH2 terminus the HA epitope in-frame with a CAG repeat either normal (17Q) or expanded (43Q) and at the COOH terminus the S65T green fluorescent protein (GFP) (5). Positive clones were selected in complete medium containing G-418, 200 μg/ml.

Cortical neurons were obtained from brains of 15–16-day-old mouse embryos as described by Abramov (7). Mouse neurons were transfected using Effectene transfection reagent according to the manufacturer’s instructions (Qiagen) with 2.5 μg of expression vectors encoding the 17 or 43Q fusion proteins described above. Human primary fibroblasts were derived from cutaneous biopsies of three HD and three SCA2 patients and three normal subjects, described in Giuliano et al. (5).

Immunofluorescence; p-H2AX in Mouse Neurons—Mouse neurons grown on glass slides were fixed in 50% methanol, 50% acetoxy for 2 h at −20 °C. After fixation, slides were washed in PBS and stained as previously described (5). Primary antibody (p-H2AX) was diluted at 1:500 in 1% bovine serum albumin, PBS and incubated overnight at +4 °C. After washing, the secondary antibody (fluorescein isothiocyanate anti-rabbit) was incubated at 1:200 in 10% FBS, PBS for 1 h at room temperature. After three washes with PBS 1×, the glass slides were mounted on coverslips. All images were captured with Axiocam microscopy (Carl Zeiss) in the same conditions of brightness and contrast.

H-Ras in Primary Fibroblasts—Primary fibroblasts grown on glass slides (Nalgene-Nunc) were starved 48 h before stimulation, then cells were fixed, permeabilized, and stained as previously described (8). All images were captured with Axiocam microscopy (Zeiss) in the same conditions of brightness and contrast.

8-Oxo-7, 8-dihydro-2’-deoxyguanosine (8-oxodG) DNA Assay on Primary Fibroblasts—For 8-oxodG detection, primary fibroblasts grown on glass slides were fixed with ice-cold 4% paraformaldehyde. After permeabilization in ice-cold methanol for 30 min, 100% of the cells were labeled as previously described (9). All images were captured with Axiocam microscopy (Zeiss) in the same conditions of brightness and contrast.

RNA Analysis—Total RNA was isolated from cells using TRI reagent according to the manufacturer’s instructions (Roche Applied Science). 1 μg of total RNA was reverse-transcribed with 100 Quantitech reverse kit (Qiagen) in a volume of 20 μl according to the manufacturer’s instructions (Qiagen).

Real-time Quantitative PCR—A quantitative assay for rat VgF8 mRNA expression was established using the GeneAmp 7900 sequence detection system (Applied Biosystems). All reactions were normalized to an endogenous control using the 129-bp fragment at 3’ 18 S mRNA. PCR oligo-primers were: Rat-VgF8 forward primer (5’-GCAGCTGAGTAT-TCTGAGAAAGG-3’) and Rat-VgF8 reverse primer (5’-CAGCAGTCTTCTTACATCAAAAATAA-3’) generating a 200-bp fragment; RAT 18 S forward primer (5’-GGCCGGCTGCTCCAGCTC-3’) and RAT 18 S reverse primer (5’-GATCGCTCTCAGTTTACTTATG-3’) generating a 200-bp fragment; and 18 S forward primer (5’- GATCGCTCTCAGTTTACTTATG-3’) and 18 S reverse primer (5’-GATCGCTCTCAGTTTACTTATG-3’). Real-time PCR was performed using the SYBR Green PCR Master Mix 2× (Applied Biosystems) and 30 ng of cDNA in a total volume of 10 μl. For VgF8 mRNA, relative quantification used the comparative Ct method according to the manufacturer’s instructions (Applied Biosystems).
Lipid Raft Fractionation; Triton X-100 Extraction—Cells treated or not with 10 mM methyl-β-cyclodextrin for 30 min at 37 °C in 100-mm dishes were washed twice with PBS containing 1 mM CaCl₂, 1 mM MgCl₂ and then lysed for 20 min in ice-cold Triton X-100 buffer (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100) (10). Lysates were collected and centrifuged at 16,000 × g for 20 min at 4 °C. Supernatants (soluble fraction) were removed, and protein concentration was measured using the Bio-Rad protein assay. The pellets (insoluble fraction) were solubilized and boiled in SDS buffer (50 mM Tris-HCl, pH 8.8, 5 mM EDTA, 1% SDS). DNA was sheared through a 22-gauge needle. Extracts were denatured and resolved by SDS-PAGE (10% or 8%) and then revealed by Western blotting (11).

Western Blot Analysis—Cells were homogenized in lysis buffer (40 mM Heps, pH 7.5, 120 mM NaCl, 5 mM MgCl₂, 1 mM EDTA, 0.5 mM EDTA, 0.6% Triton X-100) containing protease (Complete Tablets, EDTA-free, Roche Applied Science) and phosphatase (20 mM β-glycerol phosphate, 2.5 mM sodium pyrophosphate) inhibitors. Total lysate was cleared by centrifugation at 15,000 × g for 20 min at 4 °C. Protein concentration of supernatant was measured using the Bio-Rad protein assay. Extracts were denatured and resolved by SDS-PAGE (10% or 8%) gel electrophoresis (11) and then revealed by Western blotting.

Immunoprecipitation and Immunoblot—Cell lysates (1 mg) were incubated with 5–10 μg of anti-HA antibody (Roche Applied Science) at 4 °C in gentle agitation 15 h. At the end of incubation, 20 μl of A/G plus were added to the samples. After 3 h the immunoprecipitates were collected by centrifugation (1000 rpm at 4 °C). SDS-PAGE and immunoblots were performed as previously described.

Mitochondrial Membrane Potential—The ΔΨm was assessed by flow cytometry and by confocal microscopy with the fluorescent dye tetramethylrhodamine ethyl ester in the “redistribution mode” (TMRE, Molecular Probes, Invitrogen). PC12 cells expressing HA-43Q-GFP or HA-17Q-GFP were treated with AEBSF (40 μM 3 h) or apocynin (50 μM 3 h) before loading with 10 nM TMRE for 20 min in a medium containing 156 mM NaCl, 3 mM KCl, 2 mM MgSO₄, 1.25 mM KH₂PO₄, 2 mM CaCl₂, 10 mM glucose, and 10 mM HEPES at 37 °C in the dark. The pH was adjusted to 7.35 with NaOH (10). At the end of the incubation the cells were washed in the same medium containing 20 mM dye. Confocal images were obtained using a Zeiss inverted 510 confocal laser scanning microscope (Thornwood, NY) and a 63× oil immersion objective. The illumination intensity of 543 xenon laser, used to excite TMRE fluorescence, was maintained to a minimum of 0.5% of laser output to avoid phototoxicity.

Mitochondrial superoxide was measured by flow cytometry with the fluorescent MitoSOX™ Red mitochondrial indicator (Molecular Probes, Invitrogen). PC12 cells expressing HA-43Q-GFP or HA-17Q-GFP were treated with AEBSF (40 μM 3 h) or apocynin (50 μM 3 h) before loading with 5 μM MitoSOX for 10 min at 37 °C in the dark.

shRNA Plasmid Transfection—Human neuroblastoma SK-N-BE cells (American Type Culture Collection) were maintained in a mixture of RPMI 1640 medium containing 10% fetal bovine serum, 100 μg/ml glutamine, 100 μg/ml streptomycin, and 100 units/ml penicillin in 5% CO₂ at 37 °C. Cells were transfected using Metafectene according to the manufacturer’s instructions (Metafectene Transfection Reagent, BIONTEX) at 60 – 80% confluence with 2.5 μg of expression vectors coding for a fusion protein containing at the NH₂ terminus the HA epitope in-frame with a CAG repeat either normal (17Q) or expanded (43Q) and at the COOH terminus the S65T GFP (5) and 2.5 μg of pRS shRNA expression vector encoding a 29-mer shRNA against CYBB gp91 phox (OriGene technologies). Transient knockdown of CYBB was obtained using the following clones: GI354458 (sh1); GI354459 (sh2); GI354460 (sh3).

Small Interfering RNAs (siRNAs)—Lentiviral transduction particles encoding siRNA targeting H-Ras were purchased from Sigma. Fibroblasts derived from normal, HD, and SCA-2 subjects were treated with hexadimethrine bromide (8 μg/ml). Briefly, the cells were infected at a multiplicity of infection of 3.0 in complete medium (DMEM low glucose, 0.2% FCS) incubated in 6-well plates for 15 h at 37 °C in 5% CO₂. After 2 days the cells were used in the experiments. Transient knockdown of H-Ras was obtained using the following clones: NM_005343.1–108s1c1 for H-Ras. The levels of H-Ras were assayed by FACS and by fluorescence microscopy.

Statistical Analysis—All data are presented as the means ± S.E. of at least three experiments in triplicates (n ≥ 9). Statistical significance between groups was determined using Student’s t test (matched pairs test or unmatched test were used as indicated in the figure legends). For comparison of treatments in patient’s cells (n < 9) statistical significance was determined using the Wilcoxon matched test.

RESULTS

To generate a reliable experimental model for studying the impact of polyQ-expanded proteins on cellular functions, we have engineered two fusion proteins containing 1) the influenza hemagglutinin epitope (HA) at the NH₂ terminus, 2) a polyglutamine stretch (polyQ) of variable size (17Q and 43Q), and 3) a COOH tail, encoding the GFP. These proteins recapitulate the structural features of the polyglutamine expanded proteins and can be easily monitored in vivo by immunofluorescence (GFP) and immunocytochemistry (HA).

The proteins were expressed under the control of tetracycline responsive element promoter in a rat pheochromocytoma cell line, PC12, carrying an engineered version of tetracycline-controlled transactivator (tTa). The timing and the levels of the fusion proteins after the removal of the tetracycline analog doxycycline (Dox) were extensively characterized in several clones (5). Upon removal of doxycycline, the tetracycline-responsive element promoter was activated, and an intense GFP fluorescence was detected in cells transfected with the two expression vectors encoding the 17Q or 43Q fusion proteins. HA-17Q-GFP-expressing cells showed a diffuse signal throughout the cytoplasm and nucleus (5). Cells expressing the expanded polyQ protein (HA-43Q-GFP) showed initially (1–2 days after doxycycline removal) a diffuse GFP fluorescence that progressively aggregated in intra- or extranuclear spots. After 6–10 days, aggregates were present...
in 100% of the cells (5). The levels of recombinant proteins were measured in isolated clones before or after doxycycline (supplemental Fig. S1). To minimize the insertional effects on the expression of the recombined proteins, pools of clones were analyzed.

Early Events Induced by PolyQ-expanded Proteins; Mitochondria—Mitochondrial dysfunction has been associated with neurodegeneration in polyQ-expanded diseases, but the molecular link with the polyQ proteins is not known (12). To find a relation between the expression of polyQ proteins and mitochondria, we analyzed two mitochondrial markers, mitochondrial ROS and the mitochondrial transmembrane gradient (ΔΨm), at various times after doxycycline removal. Expression of 43Q protein was accompanied by rise of total cellular ROS levels at 3–6 h; later (24 h to 2 weeks), ROS levels decreased but remained higher compared with the levels found in 17Q-expressing cells (Fig. 1A). Mitochondrial ROS, measured as MitoSOX fluorescence, on the other hand, did not change in cells expressing 17Q or 43Q proteins for 3 h (Fig. 1B). The mitochondrial transmembrane gradient, assessed by fluorescence of the mitochondrial-specific rhodamine derivative, TMRE, was significantly reduced in cells expressing for the 43Q protein 3 h (Fig. 1B). Because reduction of the intensity of the fluorescent signal indicates a dissipation of the mitochondrial membrane gradient (13), we conclude that 3-h expression of the 43Q protein is sufficient to induce mitochondrial dysfunction without significant changes in mitochondrial respiration.

Another cell compartment affected by polyQ-expanded proteins is the ER (14–17). To test the function of the ER in cells expressing the 43Q protein, we analyzed the unfolding protein response after different periods of polyQ protein expression. Briefly, the unfolding protein response is activated by accumulation of unfolded proteins in the ER; the ribonuclease IRE1 removes a 26-nucleotide intron in the mRNA encoding the transcription factor XBP1, which leads to a translational frameshift to produce a new fusion protein. The new carboxyl terminus of the protein, encoded by the spliced XBP1 mRNA (XBP1-s), converts XBP1-u (unspliced) into a stable and active transcription factor which transcribes several genes that enhance degradation of misfolded proteins (18). We analyzed the accumulation of the spliced XBP1 RNA in cells expressing polyQ-expanded proteins. Fig. 1C shows the time course of expression of 43Q and 17Q fusion proteins and the accumulation of the short or the long XBP1 transcript. The

FIGURE 1. PolyQ-expanded proteins induce an early mitochondrial dysfunction and a late ER stress. A, rapid induction of ROS and mitochondrial dysfunction by 43Q polyQ-expanded proteins is shown. Total ROS were measured in PC12 cells in the presence or absence of doxycycline (3, 6, and 18 h). The expression of 17Q or 43Q was monitored by Western blot or immunofluorescence (Ref. 1 and below). PC12 clones carrying polyQ-expressing vectors under the control of tetracycline repressor were maintained in the presence of doxycycline. The induction of the fusion proteins was extensively characterized by immunoblot (HA) and fluorescence (GFP) analysis (5). ROS levels were measured at different times after the removal of doxycycline by fluorescence of the oxidation-sensitive probe, 2,7-dichlorofluorescein diacetate (DCF). The values reported are the means ± S.E. derived from at least 3 independent experiments performed in triplicate (n = 9). Differences between treatment at the given time points were tested for statistical significance using Student’s matched t test. *, p < 0.01 compared with the basal (43Q, −Dox, time 0). B, mitochondrial ROS and the mitochondrial membrane gradient were measured with the mitochondrial superoxide indicator, MitoSOX, and with TMRE, respectively, as described under “Experimental Procedures.” Differences between 17Q and 43Q were tested for statistical significance using Student’s t test. *, p < 0.01 of t value as compared with the 17Q, −Dox. The experiments were performed in cells grown for 3 h in the absence or presence of doxycycline. The concentration and the time of incubation with MitoSOX and TMRE were optimized by FACS analysis. C, time course of ER stress after polyQ-expanded protein expression is shown. ER stress was assayed by analyzing XBP-1 accumulation by RT-PCR. RNA was isolated from PC12 expressing 17Q or 43Q from 3 h to 6 weeks as indicated in the upper part of the figure on each lane. The two arrows on the right indicate the bands corresponding to the unspliced and spliced XBP-1 RNA. RT-PCR was performed using total RNA extracted from cells treated as described under “Experimental Procedures.”
short XBP1 mRNA accumulated only after one week of continuous expression of the 43Q protein. These data indicate that ER stress is a late event induced by polyQ-expanded proteins, in contrast to the rapid dissipation of the mitochondrial electrochemical gradient and the rise of total ROS, observed after 3 h of 43Q expression.

The data in Fig. 1 do not clarify if the rise of ROS levels induced by the 43Q protein is a consequence or the cause of the mitochondrial gradient dissipation. To address this point, we inhibited cellular ROS production with several oxidase inhibitors and measured the mitochondrial gradient in cells expressing polyQ proteins for 3 h and 1 day. We found that 3 h...
of treatment with 40 μM AEBSF, a NADPH oxidase inhibitor (19), reduced ROS and completely restored the electrochemical gradient in cells expressing the 43Q protein (Fig. 2A). AEBSF inhibits the binding of the p47 cytosolic subunit to the membrane gp91 NADPH oxidase complex (19), but AEBSF is also a serine protease inhibitor, and its effect may be not oxidase-specific. To test the specificity of oxidase inhibition, we challenged the cells expressing the 43Q protein with other drugs inhibiting NADPH oxidase with different mechanisms. Fig. 2B shows that apocynin prevented dissipation of the mitochondrial gradient induced by 3 h of expression of 43Q. Because ROS derived from NADPH oxidase activate ERK1/2 (20), we measured total pERK1/2 in cells expressing 43Q or 17Q proteins in the presence of diphenyleneiodonium chloride, a general NADPH oxidase inhibitor. Supplemental Fig. 2S shows that diphenyleneiodonium chloride inhibits
Common Pathogenic Trait of PolyQ-expanded Proteins

pERK1/2 only in 43Q cells, indicating that pERK1/2 in these cells were maintained active by high ROS levels. Taken together, these data suggest that the source of ROS induced by the 43Q protein is NADPH oxidase; ROS, progressively (3–6 h), target the mitochondria and dissipate the electrochemical gradient.

Oxidative DNA Damage in Neurons and Fibroblasts, Expressing a Prototypic PolyQ-expanded Protein or Mutated Huntingtin and Ataxin-2—ROS induced by the 43Q protein may target the nucleus and damage DNA. Expanded (43Q) polyQ proteins or Huntingtin has been shown to induce DNA damage, which precedes the appearance of visible aggregates (5, 21). The data shown above suggest that DNA damage signatures may be caused by ROS induced by polyQ-expanded proteins. To find a molecular link between DNA damage and polyQ proteins, we expressed 17Q and 43Q proteins in primary mouse neurons and analyzed the accumulation of p-H2AX, a substrate of the DNA damage checkpoint kinase, ATM. H2AX phosphorylated by ATM accumulates in discrete chromosomal-damaged sites (p-H2AX foci). Fig. 3A shows that primary neurons expressing 43Q proteins display a significant number of p-H2AX foci; with the same levels of expressed protein, cells expressing 17Q did not accumulate p-H2AX foci. p-H2AX foci were detected also in PC12 cells expressing the 43Q protein for 6 h and were suppressed by a generic ROS scavenger, N-acetylcysteine (NAC) (Fig. 2B).

Another independent proof of oxidative DNA damage, induced by Q-expanded proteins, is the presence of 8-oxodG foci in the nuclei of fibroblasts, derived from HD or SCA patients. The foci disappear in cells pretreated with NAC (Fig. 3C).

To find a direct link between the expression of polyQ-expanded proteins and DNA damage, we measured the accumulation of phosphorylated ATM substrates, including p-H2AX, in cells pulsed with doxycycline. Briefly, the drug was removed for 24 h and re-added (Fig. 3D, arrow) for various periods of time. Removal of doxycycline for 36 h induced accumulation of 43Q protein and activation of ATM. Re-addition of reduced doxycycline progressively polyQ proteins and prevented ATM activation (Fig. 3D). Although the turnover of p-H2AX was slower than that of phosphorylated ATM substrates, activation of ATM was tightly linked to the levels of polyQ proteins in the cell. Thus, reduction of 43Q protein levels abolished DNA damage signatures, indicating that the DNA damage was reversible and strictly dependent on the continuous presence of 43Q protein.

These experiments establish a direct link between polyQ and oxidative DNA damage. Also, these data imply that the presence of proteins carrying polyQ expansions per se induce oxidative DNA damage independently of the protein or cell context, where the expansion is located or expressed, respectively. In fact, expanded Huntingtin or Ataxin-2 or the synthetic protein fusions showed comparable phenotypes in terms of 8-oxodG accumulation and DNA damage. Collectively, these data indicate that 43Q protein induces an early ROS rise that translates into mitochondrial dysfunction, DNA damage, and activation of ATM.

Early DNA Damage Inhibits Transcription of Neural-specific Genes and Impairs the Adaptive Response to Stress and cAMP—The persistent oxidation wave, triggered by the 43Q-expanded protein, may significantly alter the growth and survival of the cells. We have extensively analyzed the growth, viability, and stress response of single or pool of clones of PC12 cells expressing 43Q protein for 3 days in the presence of NGF (supplemental Fig. S1 and Ref. 5). These cells did not significantly differ from control cells except for an impaired response to oxidative stress (5, 22). However, in neuronal terminal-differentiating cells, the absence of DNA replication may exacerbate the effects of DNA damage. Transcription of neural-specific genes is altered in polyQ-expanded diseases, and polyQ-expanded proteins may directly affect transcription. To this end we analyzed the expression of differentiation genes in cells expressing 43Q or 17Q upon treatment with NGF. Specifically, we determined the expression of the NGF-dependent gene, VgF8, by quantitative PCR in cells expressing 17Q or 43Q for 6 h (Fig. 4A). NGF induction of VgF8 was less efficient in 43Q + Dox relative to 17Q + Dox due to the age of the clone analyzed (see the legend of Fig. 4A). Down-regulation of VgF8 mRNA started 3–6 h after 43Q protein expression and persisted for weeks. Inhibition of neural-specific genes by polyQ-expanded proteins has been reported to be dependent on the formation of aggregates, which sequester...
Common Pathogenic Trait of PolyQ-expanded Proteins

Inhibition of transcription by polyQ-expanded proteins is dependent on the DNA damage checkpoint. A, VGF8 expression is silenced in 43Q expressing cells. 17Q- and 43Q-expressing cells were stimulated with NGF in the presence or absence of doxycycline. Kudos, the ATM inhibitor, was added to the cultures for 15 h. VGF8 mRNA was determined with NGF in the presence or absence of doxycycline. Kudos, the ATM inhibitor, was effective in cells expressing 43Q for 6 h, but it was very effective in cells expressing 43Q for 6 days (Fig. 4, A and B). Trichostatin, the HDAC I-II inhibitor, was effective in cells expressing 43Q for 6 h and 6 days (Fig. 4C). Because ROS have been shown to activate HDAC (26), the best interpretation of these data is that DNA damage and ROS activate ATM and HDAC. ATM also activates HDAC I (27) and may contribute to maintain active HDAC. Silencing of VGF8 is the result of these combined signals, activated by polyQ-expanded proteins.

The inhibitory effect on transcription exerted by polyQ-expanded proteins appears to be broad and also involving nonneural-specific genes. Several genes activated by stress or cAMP have been found silenced in studies analyzing the transcriptome in Huntington patients-derived cells. We have analyzed the expression of aldose reductase gene, which is stimulated by cAMP (28). In one week of continuous expression, 43Q-expressing cells progressively and selectively lost the ability to accumulate aldose reductase gene mRNA in response to cAMP (supplemental Fig. S3).

Cells expressing 43Q progressively reduce transcription, including transcription of neural-specific genes and cAMP-induced genes (29, 30). The primary cause seems to be a robust DNA damage response induced by ROS (Figs. 1 and 3). This response, orchestrated by ATM/ATR, is initially protective, as several factors (including SP1) are phosphorylated by ATM and are essential for the adaptive response to DNA-damaging agents (31, 32). Eventually, persistent ATM activation and formation of large aggregates containing transcription factors permanently impair the efficiency of the transcription machine. It is noteworthy that some cAMP-dependent genes (such as aldose reductase gene or PGC-1, the mitochondrial co-activator) can be silenced by ATM (supplemental Fig. S4 and Refs. 33 and 34), and this may amplify the mitochondrial dysfunction induced by polyQ-expanded proteins.

PolyQ-expanded Proteins Segregate in the Lipid Raft—The data reported above point to the membrane NADPH oxidase as the primary source of ROS induced by polyQ-expanded proteins. The gp91 subunit of NADPH oxidase is resident in the lipid raft compartment of the membrane NADPH oxidase (35). PolyQ-expanded proteins. The gp91 subunit of NADPH oxidase is resident in the lipid raft compartment of the membrane NADPH oxidase (35). PolyQ-expanded proteins may selectively target the expanded protein to this compartment. To test this hypothesis, we examined the partition of polyQ-expanded proteins in the lipid raft compartment of the plasma membrane, measured as detergent-resistant membranes (11, 36). These membrane microdomains are dynamic lipid fractions enriched in sphingolipids and cholesterol (37).
FIGURE 5. PolyQ-expanded proteins segregate in the lipid rafts and interact with the resident NADPH oxidase subunit gp91. A, neuroblastoma cells expressing 17Q and 43Q were lysed for 20 min in ice-cold Triton X-100 buffer (see "Experimental Procedures") and separated by centrifugation as supernatant (S) and pellet (P) fractions. The pellet fraction was dissolved in SDS buffer (indicated under "Experimental Procedures"). Lysates were immunoblotted with anti-HA, anti-ERK1/2 and anti-actinin antibodies (Ac) (upper panel). The histogram is derived from the analysis of cumulative data, derived from three experiments performed in triplicate (n = 9) and shows the fraction of soluble (on the left) and insoluble (on the right) polyQ proteins. Differences between groups were tested for statistical significance using Student’s t test. *, p < 0.01 as compared with the HA-17Q-GFP expressing cells.

B and C, segregation of wild type and mutant Ataxin-2 (B) and Huntingtin (C) in the lipid rafts is shown. Fibroblasts derived from controls and patients HD and SCA-2 were processed as in A except that in this case the cells were challenged for 30 min with 10 mM methyl-β-cyclodextrin (mβC) (as described under "Experimental Procedures"). Cell lysates were immunoblotted with antibodies against Huntingtin (HTT), Ataxin-2 (ATX-2), Nox subunit gp91, flotillin, ERK1/2 (right panel S) and actinin (left panel). The histograms are derived from the analysis of cumulative data derived from three experiments performed in triplicate (n = 9) and show the fraction of supernatant (left panel) and pellet (right panel) Ataxin-2 (B) or Huntingtin (C). D, interaction of gp91 with polyQ-expanded proteins is shown. Extracts derived from neuroblastoma cells stably transfected with either pCMV-HA-17Q-GFP or pCMV-HA-43Q-GFP were subjected to immunoprecipitation (IP) with HA antibody. The immunoprecipitates (1 mg) and total proteins were resolved on an SDS-10% PAGE and immunoblotted with anti-gp91 (upper panel) and anti-HA (lower panel) antibodies. Differences between groups were tested for statistical significance using Wilcoxon matched pairs test. *, p < 0.01 as compared with the controls; **, p < 0.01 as compared with methyl-β-cyclodextrin (−mβC) untreated samples.
Inhibition of NADPH oxidase reduces Ras levels and DNA damage induced by ROS in cells of SCA-2 and HD patients. A. HD or SCA-2 fibroblasts contain high levels of H-Ras, and inhibition of NADPH oxidase reduces H-Ras levels. Primary fibroblasts derived from control, HD, and SCA-2 patients were immuno-stained with anti-H-Ras antibody (as described under “Experimental Procedures”) and analyzed by fluorescence microscopy. Where indicated, the cells were treated with 0.5 mM \( \text{H}_2 \text{O}_2 \) for 15 min, 10 \( \mu \text{M} \) gp91-TaT or control (CTRL) peptides for 15 h, and 25 \( \mu \text{M} \) MG132 15 h (upper panel). The H-Ras signal was quantified by ImageJ 1.43 (NIH), and positive cells containing a signal 2 S.D. higher than controls were 90% in fibroblasts derived from HD and SCA-2 patients (lower panel). All data derive from at least three independent experiments performed in triplicate (\( n = 9 \)). Differences between groups were tested for statistical significance using Wilcoxon matched pairs test. *, \( p < 0.02 \) compared with the fibroblast derived from controls subjects; **, \( p < 0.01 \) compared with the untreated basal HD and SCA cells.

B. Inhibition of NADPH oxidase reduces DNA damage in HD and SCA-2 cells is shown. Human primary fibroblasts derived from normal, HD, and SCA-2 patients were seeded onto glass slides, fixed, and analyzed as described under “Experimental Procedures” for the presence of 8-oxodG. The cells were analyzed by fluorescence microscopy. Where indicated, the cells were treated with 0.5 mM \( \text{H}_2 \text{O}_2 \), the NADPH oxidase inhibitors (20 \( \mu \text{M} \) AEBSF for 15 h, 10 \( \mu \text{M} \) gp91-TaT and control peptides for 15 h, 20 \( \mu \text{M} \) apocynin for 15 h, and 20 \( \mu \text{M} \) DMSO for 15 h). The 8-oxodG signal was quantified by ImageJ 1.43 (NIH), and positive cells containing a signal 2 S.D. higher than controls were 85% in fibroblasts derived from HD and SCA-2 patients (left panel). All data derive from at least 3 independent experiments performed in triplicate (\( n = 9 \)). Wilcoxon matched pairs test: *, \( p < 0.02 \) compared with the basal, normal cells; **, \( p < 0.01 \) compared with the untreated basal HD and SCA cells.
and have been involved in many cellular processes from protein and lipid trafficking to cell signaling (38–40).

To clarify the relation between detergent-resistant membranes and polyQ proteins, we first analyzed the association of 17Q and 43Q with detergent-resistant membranes in neuronal cells. We extracted the proteins with Triton X-100 and separated the supernatant (S) and the pellet (P) fractions by gel electrophoresis. A significant fraction (55%) of the 43Q protein was found in the Triton X-100 pellet-able fraction (P), whereas almost 100% of the 17Q protein was in the supernatant (S) (Fig. 5A). This striking difference in Triton X-100 solubility between the 17Q and 43Q proteins may reflect a selective membrane segregation of the protein imposed by the polyQ stretch or may represent an artifact of this specific chimeric protein. To test directly if the natural expanded proteins behave similarly, we analyzed the fibroblasts derived

**FIGURE 7.** Silencing gp91 NADPH oxidase subunit inhibits selectively DNA damage induced by expression of 43Q in neuroblastoma cells. A, SK-N-BE neuroblastoma cells were transiently cotransfected with the vectors encoding HA-17Q-GFP or HA-43Q-GFP and several pRS shRNA expression vectors encoding a 29-mer shRNA against CYBB (gp91 phox) as described under "Experimental Procedures." After 48 h, 10⁶ cells was used for mRNA quantitation, and 2 × 10⁵ cells in triplicate were fixed with 4% paraformaldehyde and stained for p-H2AX, as described under "Experimental Procedures." CTRL cells transfected only with HA-17-GFP or HA-43Q-GFP, SCR cells cotransfected with scrambled sh; shRNA-1 and shRNA-2, cells cotransfected with two different shRNA against CYBB. B, the p-H2AX fluorescence intensity was determined on the polyQ expressing cells (GFP+) by ImageJ 1.43 (NIH) software. Fluorescence intensity differences between groups were tested for statistical significance using Student's t test (n = 20). *, p < 0.01 compared with untreated C1 (basal control); **, p < 0.01 compared with the basal 17Q; ***, p < 0.01 compared with the 43Q treated with scrambled shRNA.
from HD and SCA-2 patients. This experiment was performed by extracting the proteins with Triton X-100 after cholesterol-depleting of the cells. Cholesterol depletion, which solubilizes the lipid rafts, was achieved by pretreating the cells with methyl-β-cyclodextrin (10 mM methyl-β-cyclodextrin (30 min 37 °C), which solubilizes plasma membrane cholesterol. Fig. 5, B and C, show that wild type Ataxin-2 (Fig. 5B) or Huntingtin (Fig. 5C) were present mainly in the Triton X-100-soluble fraction, whereas the mutant Ataxin-2 or Huntingtin accumulated essentially in the insoluble fraction. Accordingly, the expanded proteins, not the wild type counterparts, were solubilized by methyl-β-cyclodextrin. The expanded polyQ proteins accumulated in the lipid raft fraction as efficiently as α-flotillin, a protein resident in the lipid-enriched fraction (41) (Fig. 5, B and C). In the same fraction we find gp91, the cytochrome subunit of membrane NADPH oxidase (Fig. 5C) (42). To further validate this finding, we performed methyl-β-cyclodextrin solubilization in PC12 cells expressing 43Q and 17Q polyQ proteins in the absence of doxycycline. Supplemental Fig. 54 shows that upon doxycycline removal, a significant fraction of 43polyQ protein is solubilized by methyl-β-cyclodextrin.

To our knowledge the selective segregation in the lipid rafts of polyQ-expanded proteins, both the recombinant (43Q) or native versions (Huntingtin and Ataxin-2), is the first reported biochemical feature that distinguishes the polyQ-expanded versus the native form of proteins. These data highlight a striking difference in the biochemical behavior of polyQ-expanded versus the wild type proteins. PolyQ expansion dictates the segregation of the protein to the lipid rafts. This appears to be dominant over the context of the original protein, as expanded Huntingtin or Ataxin-2 or the recombinant polyQ fusions segregate in the same membrane fraction. Note that these proteins share only the polyQ stretch.

Because the polyQ-expanded proteins localize in the lipid raft compartment of the plasma membrane, where gp91, an essential component of the NADPH oxidase complex, is also present (35), we tested whether polyQ-expanded protein co-immunoprecipitated with gp91. To this end, neuroblastoma cells expressing 17Q and 43Q were lysed, and the extracts were subjected to immunoprecipitation with anti-HA antibody. Fig. 5D shows that 43Q protein associates with gp91. Under the same conditions the 17Q protein did not react with gp91. At present, we do not know if the interaction is direct. Experiments with in vitro synthesized proteins suggest that the interaction is not direct and is mediated by the lipids.

Inhibition of Membrane NADPH Oxidase Dissolves the Nuclear Aggregates and Suppresses DNA Damage Induced by PolyQ-expanded Proteins—Co-segregation of polyQ-expanded proteins with gp91 may explain the early rise in ROS levels after the expression of the expanded proteins in PC12 (Fig. 1). Stabilization of gp91 makes the enzyme more susceptible to activation by serum or hormones and may prime the cells to apoptosis when subjected to stress (supplemental Fig. S1 and Ref. 5). We have found that activation of NADPH oxidase in primary cells increases ROS and p-ERK and results in stabilization of p21 H-Ras. H-Ras protein represents a sensor of ROS and in primary cells is induced by PDGF (8, 43). Because cells from HD or SCA2 patients contain higher ROS levels, H-Ras protein levels may be similarly increased. Fig. 6A shows that fibroblasts derived from patients HD and SCA-2 contain higher levels of H-Ras protein compared with normal controls. High H-Ras levels are induced in normal cells by hydrogen peroxide or proteasome inhibition (Fig. 6A and Ref. 8). Inhibition of NADPH oxidase with a gp91 dominant negative peptide (gp91-TAT), which enters the cell and competitively inhibits the association of the NADPH oxidase subunit, p47, with native gp91 (6, 44), reduces H-Ras levels in HD and SCA-2 patients (left panel). All data derive from at least 3 independent experiments performed in triplicate (n = 9). Wilcoxon matched pairs test: *, p < 0.02 compared with the basal, normal cells; **, p < 0.01 compared with the untreated basal HD and SCA cells.

FIGURE 8. H-Ras inhibition reverses DNA damage in HD and SCA-2 cells. Primary fibroblasts derived from control (CTRL), HD, and SCA-2 patients were challenged with siRNAs targeting H-Ras or scrambled H-Ras human sequences as indicated under “Experimental Procedures.” Immunofluorescence for 8-oxodG was carried out as described under “Experimental Procedures.” Where indicated, the cells were treated with 15 μM PDGF for 15 min. The 8-oxodG signal was quantified by ImageJ 1.43 (NIH), and positive cells containing a signal 2 S.D. higher than controls were 85% in fibroblasts derived from HD and SCA-2 patients (left panel). All data derive from at least 3 independent experiments performed in triplicate (n = 9). Wilcoxon matched pairs test: *, p < 0.02 compared with the basal, normal cells; **, p < 0.01 compared with the untreated basal HD and SCA cells.
FIGURE 9. Inhibition of NADPH oxidase dissolves nuclear aggregates. A, time-dependent expression of polyglutamine-GFP fusion proteins in inducible PC12 cell lines is shown. Phase contrast (lower) and fluorescence images (upper) of PC12 cells grown in the absence of doxycycline for the indicated times. The localization of GFP-fusion proteins was examined by fluorescence microscopy. Where indicated the cells were treated with 10 μM gp91-TAT and control peptides 15 h and 10 μM MG132 15 h. B, Western blot analysis was performed by using total extracts derived from PC12 cells expressing 17Q and 43Q (Dox+) and not expressing (Dox−). The extracts were immunoblotted with anti-HA antibody. The arrows indicate the apparent molecular weight of fusion proteins. The high molecular weight bands visible in the stacking gel appear only in extracts of HA-43Q-GFP cells induced for 6 days (open arrow in the right panel). C, the histogram shows the statistical analysis of the aggregates derived from at least three independent experiments performed in triplicate (n = 9), expressed as arbitrary units relative to the concentration of the 43Q protein. Wilcoxon matched pairs test: *, p < 0.05 compared with -Dox + gp91-TAT-expressing cells.
pression of the gp91 subunit in neuroblastoma cells expressing 17Q or 43Q proteins. Fig. 7, A and B, and supplemental Fig. S5 show that DNA damage, assayed as H2AX foci, induced by 43Q, is inhibited by down-regulating gp91 subunit of NADPH oxidase.

To establish the link between Ras levels and DNA damage in SCA-2 and HD cells, we silenced specifically H-Ras (supplemental Fig. S6) and monitored DNA damage in HD and SCA2 cells. Fig. 8 shows that DNA damage induced by polyQ-expanded Huntingtin or Ataxin-2 disappears in H-Ras-silenced cells. These data indicate that H-Ras is an essential component in the chain of events leading to DNA oxidative damage by polyQ proteins. ROS and high levels of H-Ras favor activation of ERK1/2 signaling at low levels of active receptor(s) (8). High H-Ras levels, induced by ROS, amplify the ERK1/2 cascade and allow primary cells to become extremely sensitive to growth factors stimulation and stress. In vivo, activation of specific receptors may target the burden to selected neuronal populations. These cells strive to survive by forming aggregates and activating stress kinases (43) and progressively and slowly succumb to oxidative stress.

One important question partly unanswered is the reversibility of the cellular phenotypes induced by polyQ-expanded proteins. Expression of the polyQ-expanded proteins induces the formation of nuclear aggregates, and removal of the primary cause should be able to inhibit the formation of new aggregates without dissolving the older aggregates. Because we find that activation of membrane NADPH oxidase is the earliest event induced by the polyQ-expanded proteins, prolonged inhibition of this enzyme should be able to reduce the formation of aggregates. We have measured by fluorescence microscopy the formation of aggregates in the presence of the specific NADPH oxidase inhibitor, gp91-TAT.

Fig. 9A and supplemental Fig. S7 show that 1) the aggregates were strictly linked to the expression of polyQ-expanded proteins, 2) the aggregates were visible after 7 days of continuous expression of polyQ proteins, and 3) treatment of the cells with gp91-Tat not only reduced the formation of new aggregates but dissolved the older ones. We have also analyzed the formation of the aggregates by polyacrylamide gel electrophoresis. HA-positive aggregates can be detected at the interface between the running and stacking gels (Fig. 9B). This assay shows that inhibition of NADPH oxidase with the selective interfering peptide reduces the levels of high molecular weight proteins. Collectively these data indicate that 1) activation of membrane NADPH oxidase by polyQ-expanded proteins is directly responsible for formation of the aggregates, and 2) these aggregates are continuously formed and dissolved.

DISCUSSION

The data presented here highlight a dominant function of the polyQ expansion per se. The protein containing the 43 polyQ expansion and the expanded Huntingtin or Ataxin-2 segregate preferentially in the lipid raft compartment and associate with the catalytic subunit of NADPH oxidase, gp91. Our data indicate a mechanistic link between formation of aggregates and NADPH oxidase activation.

The NADPH oxidase complex is constituted by different cytosolic subunits and by the membrane cytochrome b558 heterodimer, composed of the small p22 phox and by one of the five catalytically active NOX homologous proteins, including the phagocytic NOX2 (gp91phox) and NOX1, NOX3-NOX5. Upon activation of the complex, the cytosolic subunits translocate to the membrane and bind NOX protein. The variety of inhibitors and the silencing experiments (Fig. 7 and supplemental Fig. S5) point to NOX2 as the main target of expanded-polyQ proteins in neurons and fibroblasts, although we cannot rule out that other gp91 homologous subunits (NOX1-NOX4) resident in the lipid rafts may be targeted by polyQ-expanded proteins. This adds a novel level of complexity to the pathogenesis of the diseases induced by polyQ proteins because 1) glial cells express NOX2, and they can contribute to ROS generation that may further stimulate surrounding neurons (46), 2) neurons in specific compartments may express specific NADPH oxidases (DUOX1 in cerebellum, for example) coupled to specific receptors, and 3) most importantly, acute or persistent stimulation of specific receptors may be amplified in a subset of neurons. For example, striatal spiny neurons and cholinergic interneurons display differential ionotropic responses and vulnerability to oxidative stress (47). Together, these elements may contribute to the specificity of the effects on selected neurons, elicited by polyQ-expanded proteins (48, 49).

These data also provide a possible explanation of a long-standing problem in the pathogenesis of polyQ expansion diseases; that is, the impact of poly-Q-expanded proteins on folding of other proteins and the progressive disruption of cellular protein folding capacity in models of polyQ diseases (50). Constitutive activation of membrane NADPH oxidase and persistent, albeit low, ROS target and inhibit the catalytic subunit of the proteasome (51). This can be compensated for variable periods of time, depending on the cell type. Progressively under these conditions, the efficiency of the proteasome decays (8, 52). As consequence, the turnover of many cell proteins slows down (53). In neurons, aggregate formation and accumulation of proteins cannot be diluted by cell division, and the burden imposed on the cells increases with the time.

In conclusion, these data suggest a common pathogenic mechanism in all polyQ-expanded diseases, dependent on the ability of polyQ to interact with lipid rafts and consequently to stabilize NADPH cytochrome subunit, gp91. It remains to be seen how each neuron type reacts to this persistent oxidative signal and which are the long term consequences on the function and survival of specific networks.

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