Biochemical and Biophysical Research Communications **260,** 150–158 (1999)
Article ID bbrc.1999.0851, available online at http://www.idealibrary.com on IDEAL®

The Length of Polyglutamine Tract, Its Level of Expression, the Rate of Degradation, and the Transglutaminase Activity Influence the Formation of Intracellular Aggregates

Tiziana de Cristofaro,*[,1] Adelina Affaitati,†[,1] Lucio Cariello,† Enrico V. Avvedimento,‡ and Stelio Varrone*[,2]

*Centro di Endocrinologia ed Oncologia Sperimentale (C.E.O.S.) del C.N.R c/o Dipartimento di Biologia e Patologia Cellulare e Molecolare, Universita' di Napoli "Federico II", Napoli, Italy; †Laboratorio di Biochimica e Biologia Molecolare, Stazione Zoologica "Anton Dohrn", Napoli, Italy; and ‡Dipartimento di Medicina Sperimentale e Clinica, Universita' degli Studi di Catanzaro "Magna Grecia", Catanzaro, Italy

Received March 2, 1999

A common feature of CAG-expansion neurodegenerative diseases is the presence of intranuclear aggregates in neuronal cells. We have used a synthetic fusion protein containing at the NH_2 terminus the influenza hemoagglutinin epitope (HA), a polyglutamine stretch (polyQ) of various size (17, 36, 43 CAG) and a COOH tail encoding the green fluorescent protein (GFP). The fusion proteins were expressed in COS-7 and neuroblastoma SK-N-BE cells. We found that the formation of aggregates largely depends on the length of polyglutamine tracts and on the levels of expression of the fusion protein. Moreover, transglutaminase overexpression caused an increase of insoluble aggregates only in cells expressing the mutant expanded protein. Conversely, treatment of cells with cystamine, a transglutaminase inhibitor, reduced the percentage of aggregates. We found also that the inhibition of the proteasome ubiquitin-dependent degradation increased the formation of intranuclear aggregates. These data suggest that length of polyglutamine tract, its expression, unbalance between cellular transglutaminase activity, and the ubiquitin-degradation pathway are key factors in the formation of intranuclear aggregates.

The expansion of CAG repeats is the common genetic defect underlying eight inherited neurodegenerative disorders: Huntington's disease (HD) (1), dentatorubropallidoluysian atrophy (DPRLA) (2, 3), spinal and bulbar muscular atrophy (SBMA) (4, 5) and five spinocerebellar ataxias (SCA1, SCA2, SCA3, SCA6, SCA7) (6–11). It has been proposed that the genetic defect acts by dominant gain of function (12).

Although these diseases share genetic and molecular aspects, each disorder shows the death of specific populations of neurons, as indicated by *in vitro* and *in vivo* studies (1–12). The selective death of neurons might be caused by the inactivation of a specific neuronal factor(s) by the mutant protein or by increased sensitivity to apoptotic stimuli amplified by the mutant protein in specific neuronal populations.

A common feature of these neurodegenerative disorders is the presence of intracellular aggregates in neuronal cells (13, 14). In fact, intranuclear deposits were revealed in neuronal cells of mice transgenic for exon 1 of HD (15), for ataxin 1 (16) and in brain of HD (17), SCA1 (18), SCA3 (19) and SCA7 (20) patients. In addition intranuclear inclusions were detected in DPRLA transfected COS-7 cells (21).

It is still unclear the significance of the aggregate formation in the neurodegenerative process. The aggregates, according to a current hypothesis, are toxic for the cell survival, and commit the cell to death (22, 23). In contrast cell death without aggregate formation has been found, indicating that intranuclear inclusions might represent a survival strategy to a toxic insult caused by the mutant protein (24, 25).

Recent data have also suggested a role for transglutaminase (TGase), the enzyme which utilizes as sub-

[1] Tiziana de Cristofaro and Adelina Affaitati contributed equally to the manuscript.

[2] To whom correspondence should be addressed at Centro di Endocrinologia ed Oncologia Sperimentale (C.E.O.S.) del C.N.R c/o Dipartimento di Biologia e Patologia Cellulare e Molecolare, Universita' di Napoli "Federico II", Via S. Pansini 5, I-80131 Napoli, Italy. Fax: +39-081-7463243. E-mail: varrone@alpha.szn.it.

0006-291X/99 $30.00

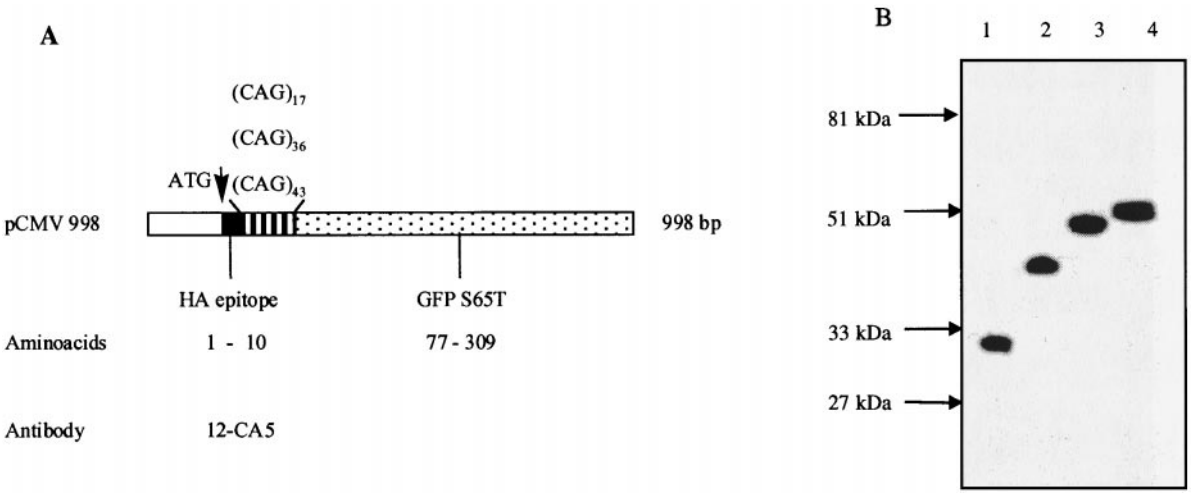

FIG. 1. Structure and expression of fusion proteins containing CAG repeats. (A) Structure of the fusion proteins containing at the NH_2 terminus the HA epitope (black box); the CAG repeats of 17, 36 and 43 units; at the COOH terminus the S65T green fluorescent protein. The details of the construction are described in Materials and Methods. The relative amino acid positions are indicated. (B) Immunoblot analysis of expressed fusion proteins in COS-7 cells (50 μg of total cell extracts) with anti-HA antibody, as described in the Materials and Methods. Lane 1: HA-GFP. Lane 2: HA-17Q-GFP. Lane 3: HA-36Q-GFP. Lane 4: HA-43Q-GFP.

strate proteins with expanded polyglutamine stretches (26–29).

In the present report we have explored the effects of glutamine repeat length on the formation of intranuclear aggregates in cultured cells overexpressing transglutaminase. We have found that the formation of aggregates is largely dependent both upon the levels of expression of the target protein and the length of polyglutamine. We show that the formation of aggregates is increased by transglutaminase overexpression and it is suppress by TGase inhibitors. Also, we present data indicating that inhibition of the proteasome increases the rate of aggregate formation.

MATERIALS AND METHODS

Plasmid construction. The HD fragments with expanded $(CAG)_n$ were generated by PCR (chain polymerase reaction) on genomic DNA, isolated from patient peripheral blood lymphocytes (30) using the sense primer 5′TGAGAATTCCGCCTTCGAGTCCCTCAAGTCCTTC3′ and the antisense primer 5′CTGGAATTCCGGCTGAGGAAGCTGAGGAG3′, both containing *EcoR* I site. PCR products were gel purified (QIAGEN), *EcoR* I digested and subcloned into pECE vector (31, 32), containing an hemoagglutinin influenza human virus epitope, containing the first methionine of the fusion protein and *EcoR* I cut at COOH terminus. The resultant vector, containing the HA- $(CAG)_n$ fragment, was digested with *Hind* III/*Xba* I enzymes.

We have amplified S65T GFP cDNA from pEGFP vector (Clontech) using the sense primer 5′CGATAAGCTTACCATGGTGAGCAAGGGC3′, containing *Hind* III site and the antisense primer 5′CTAGTCTAGACGCTTTACTTGTACAGCT3′, with *Xba* I site. The PCR product was gel purified, *Hind*I II/*Xba* I digested and subcloned into pECE-HA- $(CAG)_n$ plasmid with *Hind* III/*Xba* I cohesive ends. The resultant fusion fragments HA- $(CAG)_n$-GFP were removed from pECE vector using a *Not* I site and an *Apa* I site and ligated into a pRc/CMV vector (Invitrogen) with cohesive ends and carrying geneticin resistance. The nucleotide sequencing of all constructs was checked by Sanger dideoxynucleotide-mediated chain-termination method (30). All fusion proteins were *in vitro* translated with TNT T7/T3 coupled reticulocyte lysate system (Promega).

Cell culture and transfection. COS-7 cells, obtained from ATCC, were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL) supplemented with gentamicin (Gibco BRL) and 10% foetal calf serum (Gibco BRL). SK-N-BE neuroblastoma cells, from ATCC, were maintained in RPMI-1640 (Gibco BRL) supplemented with gentamicin (Gibco BRL) and 16% foetal calf serum (Gibco BRL). Cells were transfected with 5 μg DNA using the calcium phosphate precipitation method (30). Purified DNA was prepared using QIAGEN tips columns (Qiagen, Chatsworth, CA). Precipitates were added to cells and incubated at 37°C in a 5% CO_2 incubator for 16 hours. Then fresh medium was added to the monolayer. Cells were incubated in 250 μM and 500 μM cystamine (Sigma) (33, 34) for 96 hours. Lactacystin (Calbiochem) (35) treatment was performed at 10 μM final concentration for 48 hours. To isolate neuroblastoma cells stable transfectans it has utilized geneticin 800 μg/ml (Gibco BRL). Transfected cells were washed twice in phosphate-buffered saline (PBS). Cells were examined under phase and fluorescence microscopy (Axiovert 100S Zeiss) with 495-nm filter. The percentage of aggregates were determined by counting cells with visible aggregates, divided by the total number of green fluorescent cells (500 transfected cells) 48 hours, 72 hours and 96 hours after transfection, in three independent experiments.

Incubation of HA-poly-glutamine protein with transglutaminase. Transfected cells were washed once in phosphate-buffered saline (PBS), scraped in 40 mM Tris-HCl, pH 7.5, 1 mM ethylenediaminetetraacetate (EDTA) and 150 mM NaCl and centrifuged. The cell pellet was suspended in 50 mM Tris-HCl, pH 7.5, and sonicated for 20 s. 700 μg of proteins were incubated in the presence of 10 mU of purified guinea pig liver transglutaminase (Sigma) in 210 μl of buffer containing 10 mM $CaCl_2$, according to Kahlem *et al.* (1998) (28). Samples of 30 μl were taken at 0, 1 hour and 3 hours time and EDTA was added to 70 mM to stop the reaction. Same experiments were

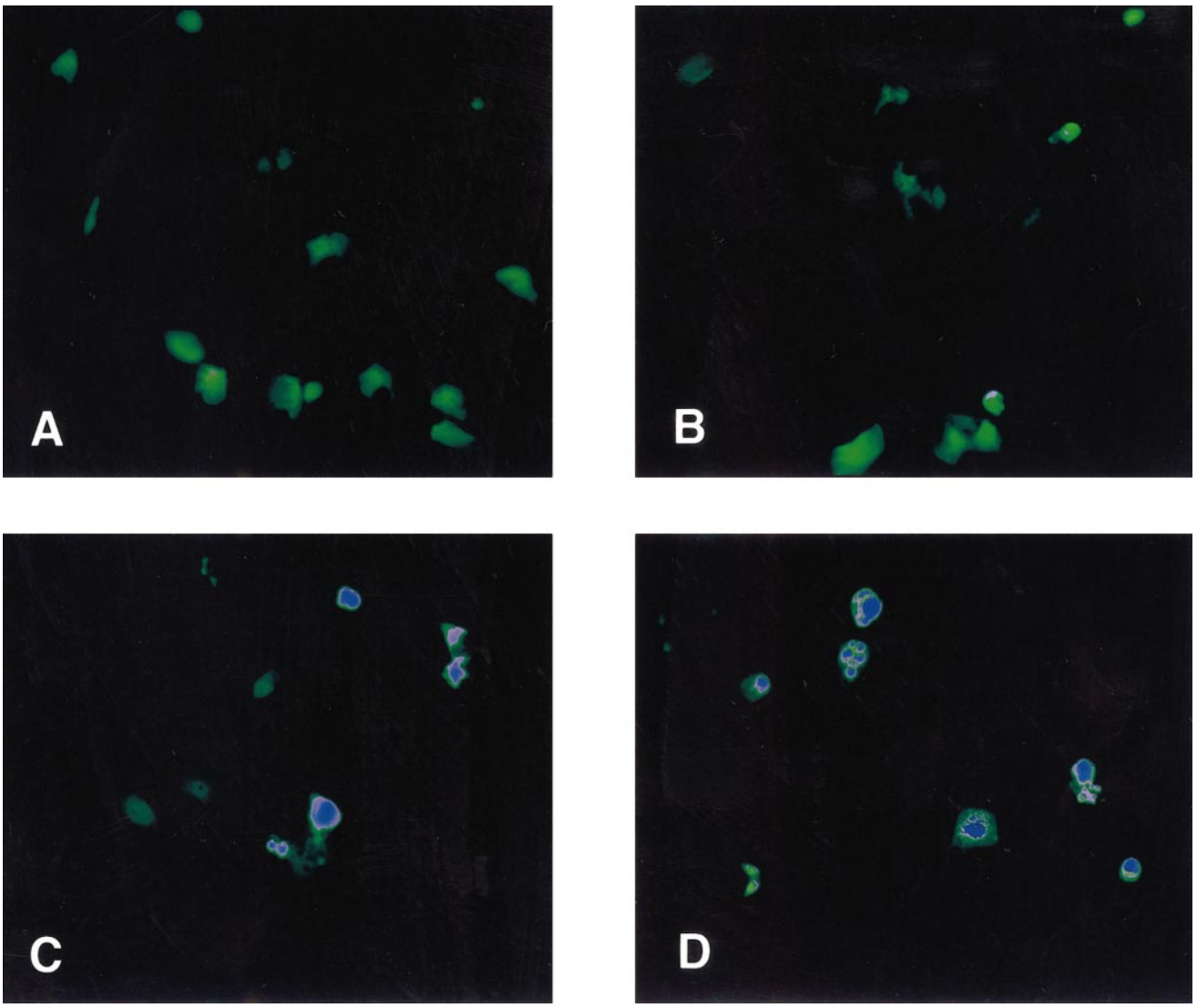

FIG. 2. Fluorescence analysis of the HA-polyglutamine-GFP fusion products in transfected COS-7 cells. Cells were transfected with 5 μg of an expression vector encoding the HA- $(CAG)_n$-GFP fusion products described in the text. 96 hours after transfection the cells were analyzed. Panels A, B, C and D show the cells expressing the control, 17-, 36-, 43-Q-GFP fusion proteins, respectively. The purple-violet signals indicate areas with high levels of fluorescence relative to an arbitrary threshold.

performed in the presence of 70 mM EDTA and 10 mM cystamine. The protein concentrations were determined (BIO-RAD protein assay) (36). The reaction products were subjected to immunoblot analysis, with anti-HA monoclonal antibody.

Immunoblot analysis. 40–80 μg of total proteins in 0.5 M Tris-HCl, pH 6.8, 2% SDS, 0.05% BBF, 100 mM DTT and 20% glycerol were boiled for 5 min and than separated on 12.5% SDS-PAGE. Proteins were transferred to PVDF membranes (Schleicher & Schuell) at 250 mA for 80 min. The blots were blocked in 10% non-fat dry milk, 10 mM Tris-HCl, pH 8.8, 50 mM NaCl and 0.1% Tween 20 (TBS-T) overnight at 4°C. (a) Immunoblot with anti-HA monoclonal antibody (12CA5 Boehringer Mannheim): blots were incubated with 1:1000 dilution of anti-HA antibody 2 hours at room temperature in TBS-T, 5% milk. Blots were washed for 2 × 15 min and 2 × 5 min with TBS-T, 5% milk. They were incubated with 1:3000 dilution of Goat anti-mouse F(ab′) 2-HRP conjugated (Amersham) for 1 hour at room temperature. After washing (2 × 15 min, 2 × 5 min with TBS-T, 5% milk and 2 × 15 min with TBS-T), blots were developed using the enhancer chemiluminescence reagent (ECL kit, Amersham). (b) Immunoblot with anti-guinea pig liver (GPL) transglutaminase polyclonal antibody provided by L. Lorand. Blot was incubated with 1:1000 dilution of anti-GPL transglutaminase antibody 1 hour at room temperature in TBS-T, 5% milk. Blot was washed for 2 × 15 min and 2 × 5 min with TBS-T, 5% milk. It was incubated with 1:3000 dilution of anti-rabbit F(ab′) 2-HRP conjugated (Amersham) for 2 hours at room temperature. After washing (2 × 15 min, 2 × 5 min with TBS-T, 5% milk and 2 × 15 min with TBS-T), blot was developed using the enhancer chemiluminescence reagent (ECL kit, Amersham).

Transglutaminase assay procedure. TGase activity was analyzed on protein extracts as previously described (Lorand *et al.*, 1972) (37). The activity was measured as picomoles of labeled putrescine (Amersham) incorporated/min/protein.

RESULTS

To generate a reliable model for the analysis of the biological consequences of the expression of proteins carrying CAG repeats under controlled conditions, we have constructed a fusion protein containing at the NH_2 terminus the influenza hemoagglutinin epitope (HA), a polyglutamine stretch (polyQ) of various size (17, 36, 43 CAG) and a COOH tail encoding the green fluorescent protein (GFP) (Fig. 1A). This protein recapitulates the features of the HD alleles and can be easily monitored and assayed by fluorescence (GFP) and immunohystochemistry (HA). The synthetic genes were subcloned in a mammalian expression vector containing the CMV promoter and expressed in COS-7 cells. The fusion proteins of the expected molecular weight, depending on the presence of 17, 36 and 43 CAG repeats, were efficiently expressed in COS-7 cells (Fig. 1B).

Length and Expression Levels of the Polyglutamine Tracts Influence the Frequency of Formation of Intracellular Aggregates

Cells expressing HA-polyglutamine-GFP fusion proteins were examined by fluorescence microscopy 48 hours, 72 hours and 96 hours after transfection. Figure 2 shows that both the HA-GFP (A) and the HA-17Q-GFP (B) transfected cells presented a diffuse green fluorescence uniformly distributed throughout the cytoplasm and the nucleus. In contrast 48 hours after transfection both the HA-36Q-GFP (C) and the HA-43Q-GFP (D) fusion proteins formed large aggregate bodies, which were localized in the nucleus. The fraction of cells containing intranuclear aggregates dramatically increased 72 hours and 96 hours after transfection. The intranuclear aggregates were present only in cells expressing the 43-polyQ-fusion protein and at lesser extent in the cells containing the 36-polyQ protein. Figure 3 shows that there was a linear relation between the time after the transfection and the fraction of cells containing intranuclear aggregates. The maximal expression of transfected plasmid occurred between 48 and 72 hours after transfection, indicating that the formation of the aggregates was critically dependent upon the levels of protein expressed. Intracellular and intranuclear aggregates were never detected with the HA-GFP and the HA-17Q-GFP expression vectors, independently on the level of expression (Fig. 3 and data not shown). The protein containing 36-polyQ formed aggregates at high DNA concentration and less efficiently than the 43-polyQ protein (Fig. 3). Note that the level of expression of the fusion proteins analyzed here by immunoblot with anti-HA antibodies was comparable (Fig. 1B). Also, we did not detect cleavage products containing HA epitope in the various transfections (data not shown).

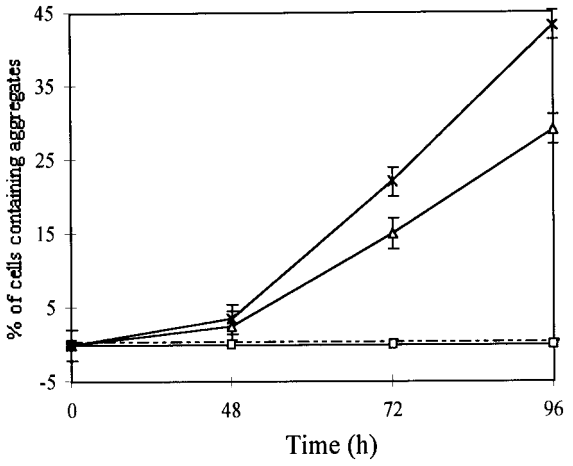

FIG. 3. Time-course of aggregate formation in transiently transfected COS-7 cells. The aggregates were determined by counting 500 cells per sample in triplicate as described in Materials and Methods. The aggregates correspond to the nuclei. HA-GFP (- - -), HA-17Q-GFP (□), HA-36Q-GFP (△), HA-43Q-GFP (×).

We next determined whether the presence of intracellular aggregates in HA-43Q-GFP expressing cells resulted in death of these cells. Staining of cells at 48, 72 and 96 hours after the transfection with propidium iodide or annexin indicated that the cells containing the fluorescent aggregates were alive and did not present anticipatory signs of apoptosis. Rare apoptotic cells were detected in transfected cells, but no direct correlation between intracellular aggregates and apoptosis was found (data not shown).

To determine the long-term effects caused by the expression of the constructs illustrated above, we have generated stable transfectants in SK-N-BE neuroblastoma cells. Only cells expressing the 43-polyQ protein showed the presence of intranuclear aggregates (Fig. 4). Moreover, 43-polyQ expressing cells after 10-15 cycles did not grow and died. Only cells expressing very low levels of fusion protein survived. These cells did not show aggregates (data not shown). These data suggest that the aggregates formed by the HA-43Q-GFP fusion protein affect the long-term survival of the cells.

Expression of Transglutaminase Increases the Frequency of Intracellular Aggregates

We have transiently expressed in COS-7 cells the human transglutaminase (tTGase) cDNA driven by SV40 promoter. Determination of TGase activity in transfected and control cells indicated that expression of the exogenous gene substantially stimulated the activity of the enzyme (Fig. 5A). The increased activity was dependent on the levels of the protein as shown by the immunoblot illustrated in Fig. 5B. Co-expression of tTGase with the fusion proteins carrying the polyQ

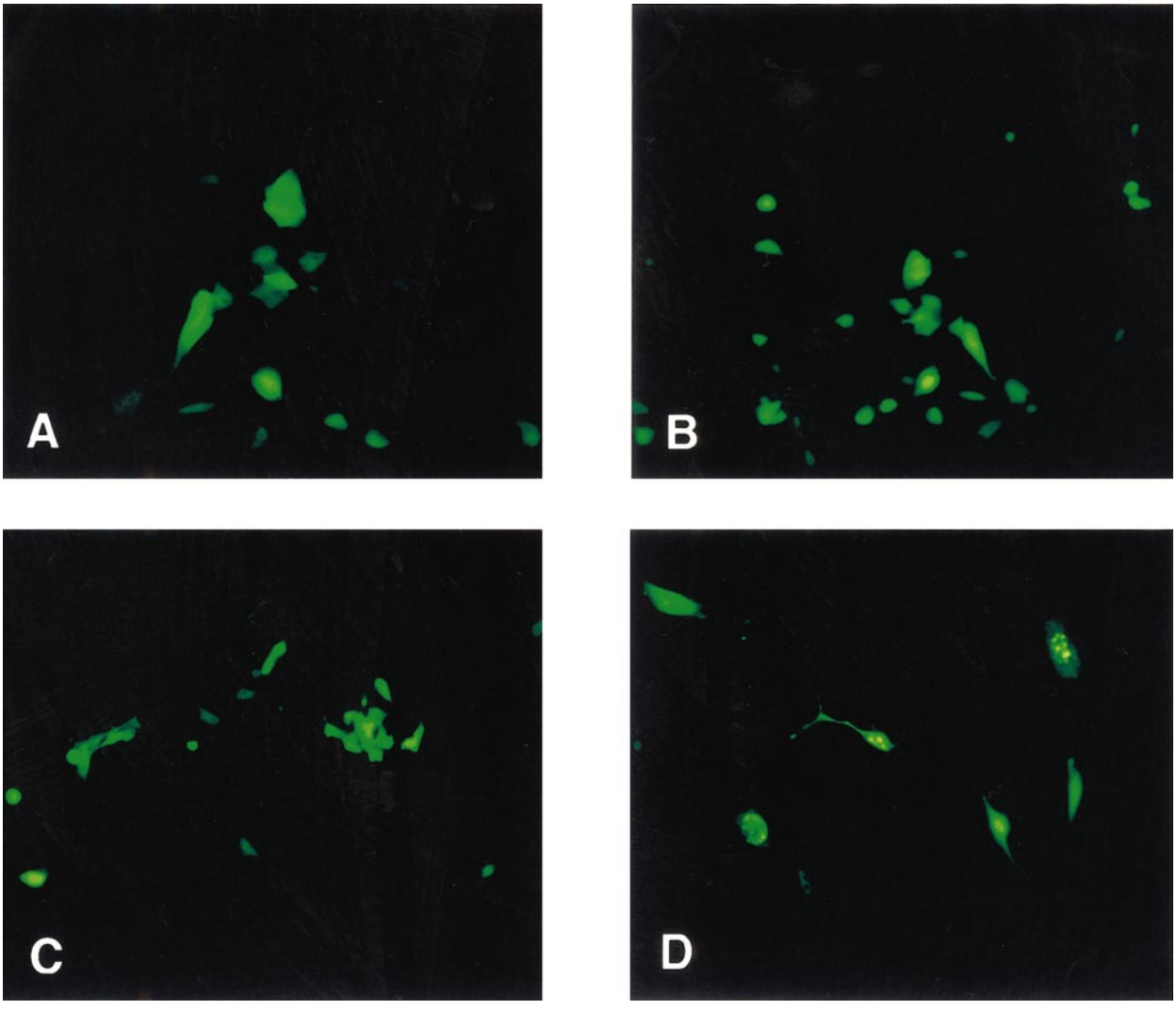

FIG. 4. Stable expression of CAG repeats fusion products in SK-N-BE neuroblastoma cells. Cells were transfected with 10 μg of an expression vector encoding the HA-polyQ-GFP fusion products described in Fig. 1 and the neomycin resistance gene. Stable transfectants were selected and pools of ca. 50 clones per construct were further analyzed. Expression analysis of the fusion protein did not show substantial differences among the pools of clones. Fluorescence analysis of the cell lines expressing the control, the 17-, 36- and 43Q-GFP fusion proteins (panel A, B, C, D, respectively) revealed the presence of intranuclear aggregates exclusively in cells expressing the 43CAG-GFP fusion proteins (25% $\pm$ 0.5% in 1000 cells analyzed). The growth of the HA-43Q-GFP expressing cells was severally reduced (data not shown).

resulted in a substantial increase of the intranuclear bodies in cells expressing 36- and 43-polyQ (Fig. 5C). The aggregates were never found in cells expressing the 17-polyQ protein, HA-GFP and tTGase (data not shown).

To demonstrate the specificity of transglutaminase effect on the formation of intracellular aggregates, we have incubated HA-43Q-GFP transfected cells in the presence of cystamine, an inhibitor of transglutaminase. The cystamine treatment reduced tTGase activity (data not shown) and the formation of intracellular aggregates both in tTGase transfected and control cells. Note that 500 μM cystamine treatment decreased the frequency of intracellular aggregates from 54 to 19,5% in cells overexpressing transglutaminase (Fig. 5D).

We have also incubated extracts from cells expressing the HA-43Q-GFP fusion proteins with purified guinea pig liver transglutaminase and analyzed by immunoblot with anti-HA antibodies. *In vitro* tranglutaminase substantially increased the multimeric bands containing the HA epitope. The HA-43Q-GFP protein was a very efficient substrate, since the monomeric band completely disappeared (Fig. 6, lanes 1, 2, 3). Under the same conditions the enzyme (data not shown) did not efficiently utilize the HA-36Q-GFP fu-

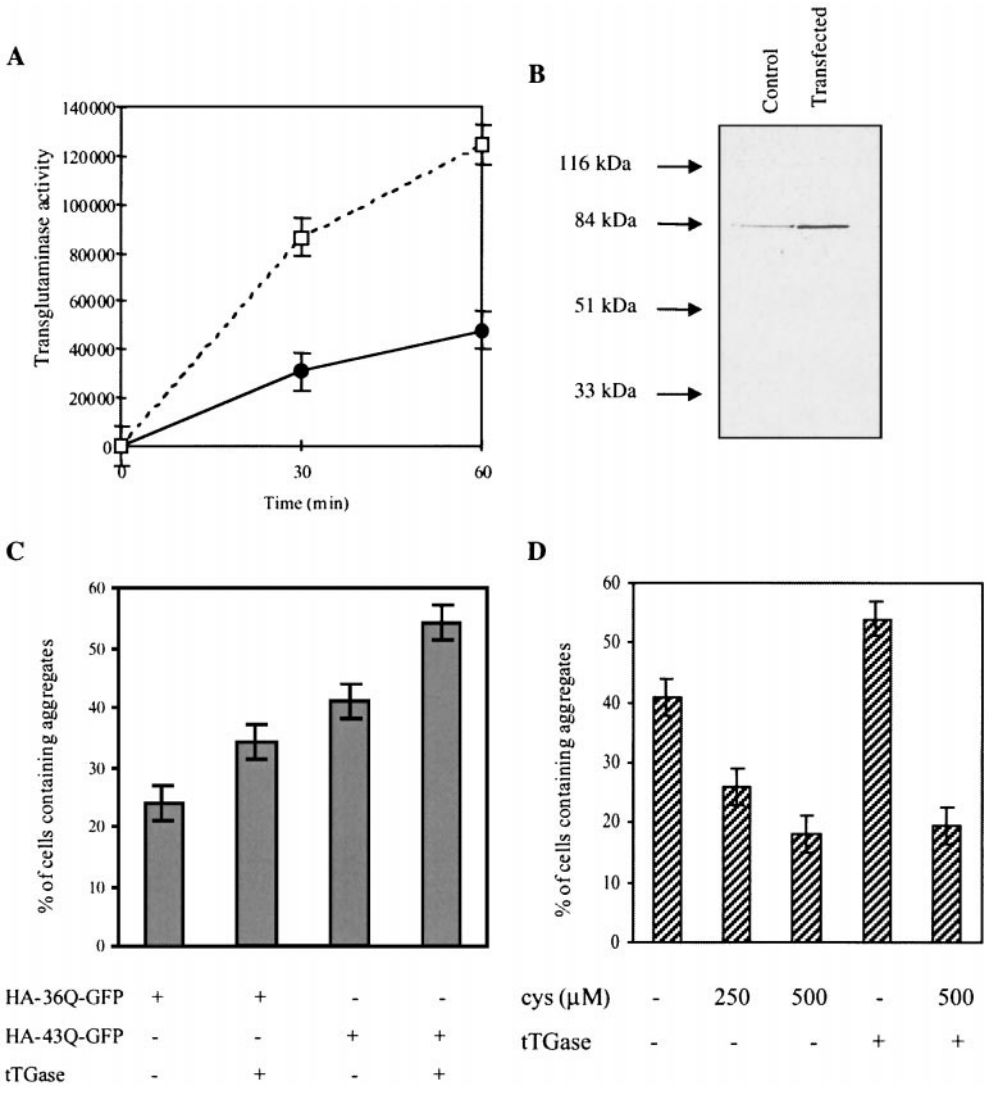

FIG. 5. Transglutaminase expression stimulates the formation of intranuclear aggregates. (A) Transglutaminase activity in control (●) and cells transfected with the human tranglutaminase cDNA (□), as described in Materials and Methods. The activity is represented as picomoles of ^{14}C-putrescine incorporated/min/mg protein. (B) Levels of transglutaminase by immunoblot analysis in 50 μg of total cells extracts from control and transfected cells. (C) Frequency of aggregates in cells expressing the 36- and 43-polyQ fusion proteins and exogenous transglutaminase. (D) The transglutaminase inhibitor, cystamine, decreased the frequency of aggregate formation in 43-polyQ fusion protein expressing cells. Transfected cells were treated with the indicated concentrations of cystamine for 96 hours. Cells transfected with exogenous transglutaminase are indicated.

sion protein. These effects were specifically dependent on transglutaminase, since they were inhibited by EDTA (data not shown) and by cystamine (Fig. 6, lanes 4, 5, 6).

Inhibition of Ubiqutin-Mediated Degradation Increases the Frequency of Intracellular Aggregates

We next tested whether the inhibition of ubiquitin degradation pathway influences the frequency of formation of intranuclear aggregates. We have incubated HA-polyQ-GFP transfected cells with lactacystin, a powerful inhibitor of the proteasome. Immunoblot analysis with HA antibody indicated that the multimeric bands containing the HA-43Q-GFP fusion protein substantially increased following 48 hours treatment with the drug (Fig. 7A). Figure 7B shows that 48 hours treatment with the drug markedly increased the frequency of intranuclear aggregates.

DISCUSSION

The expansion of CAG repeats encoding polyglutamine stretches causes several neurodegenerative

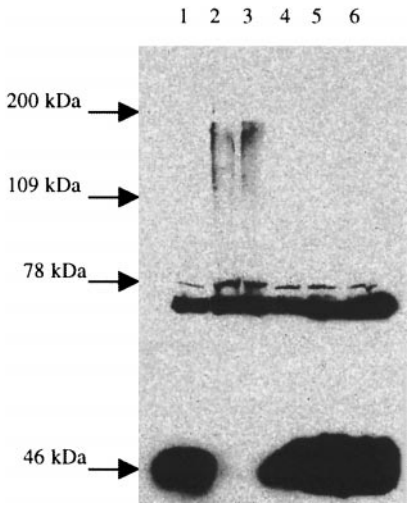

FIG. 6. The HA-43Q-GFP fusion protein is a very efficient substrate of transglutaminase *in vitro.* Extracts (40 μg) of cells expressing the HA-43Q-GFP fusion protein were treated with 10 mU of guinea pig liver TGase for 0, 1 and 3 hours at 37°C without (lanes 1, 2 and 3) and with cystamine (lanes 4, 5 and 6). After the reaction, the proteins were blotted and probed with anti-HA antibodies. ECL (30 s exposure) of the filter is shown. Protein aggregates were also detected in the stacking gel (data not shown).

disorders probably through a common mechanism. The type and the timing of neuronal loss are highly specific and partly dependent on the size of the expanded CAG repeat. Under some circumstances, the expanded protein forms aggregates, which might act as seed inducing cell death. Intracellular aggregates are the distinctive feature of the pathological allele in cells expressing the expanded protein. Although there are cases of neuronal death without the presence of aggregates, the pattern and the frequency of the aggregate represents the best-characterized experimental phenotype expressed by the mutated gene (22–25). The mechanism(s) causing protein aggregation is unknown, but one possibility is that the normal protein conformation is destabilized by the presence of the expanded polyglutamine tract, which leads to abnormal protein-protein interactions, formation of nuclear aggregates and interference with essential nuclear functions.

The fusion proteins that we have analyzed recapitulate the features of the normal, pre and pathological HD alleles. Thus, the HA-43Q-GFP fusion protein forms intranuclear aggregates more efficiently than the HA-36Q-GFP fusion protein. The HA-GFP and HA-17Q-GFP protein never formed inclusion bodies. Our data indicate that the factors influencing the formation of the aggregates are:

1. CAG length and expression. We have shown that the formation of the aggregates largely depends on the levels of expression of the protein. The aggregates are formed in COS-7 cells 48-72 hours after transfection, a period corresponding to the maximal expression of the protein. We have also performed DNA dose-response curves of all the fusion proteins analyzed here, and only 43-polyglutamine repeats containing protein and at lesser extent 36-polyglutamine protein formed ag-

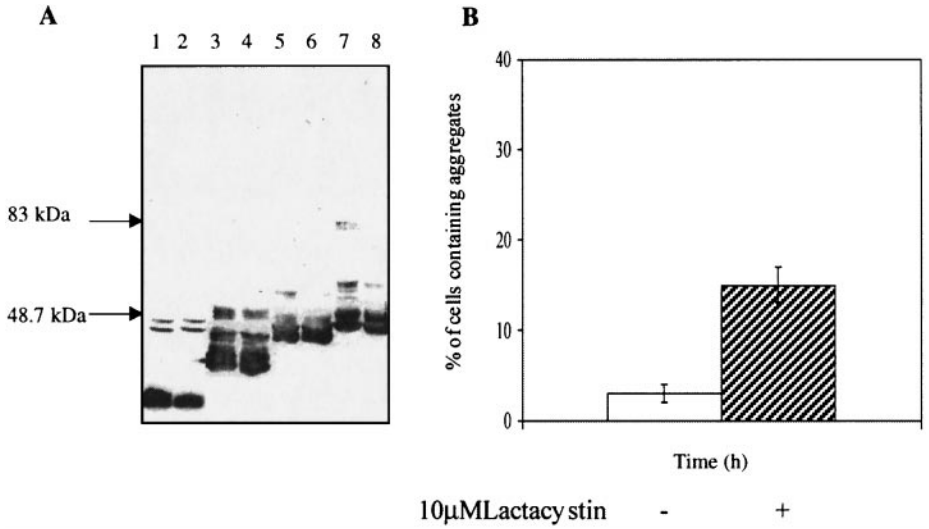

FIG. 7. Lactacystin stimulates the formation of aggregates in HA-43Q-GFP expressing cells. (A) Immunoblot analysis of protein extracts (40 μg) from HA- $(Q)_n$-GFP transfected cells treated with lactacystin (10 μM) for 48 hours. Lanes 2, 4, 6 and 8 indicate control, 17-, 36- and 43-polyQ fusion proteins extracts without lactacystin. Lanes 1, 3, 5 and 7 indicate the same samples derived from cells treated with lactacystin. The multimeric bands, containing the HA-43Q-GFP fusion protein (indicated by the major band of ca. 83kDa molecular weight), increased following 48 hours incubation with the lactacystin. (B) Histogram shows the frequency of aggregate formation in HA-43Q-GFP expressing cells after 48 hours of lactacystin treatment.

gregates. The failure of 17-polyglutamine protein to form aggregate was not dependent on the levels of expression, since immunoblot analysis showed comparable levels of the polyQ containing proteins. Also, the efficiency of aggregate formation did not appear to be dependent on the method of transfection used (transient versus stable).

2. Transglutaminase activity. The data presented above indicate that there is a critical threshold of the number of CAG repeat triggering the formation of intracellular aggregates. Polyglutamine is an excellent substrate of transglutaminase (38). There are data suggesting that transglutaminase might be involved (39). Figure 6 shows that the 43-polyQ containing protein was an excellent substrate for tTGase, since the band corresponding to the monomeric protein completely disappeared when exogenous TGase was added to the reaction. Under the same conditions the enzyme did not efficiently use the 36-polyQ containing protein. These data might explain the difference in the frequency of aggregate formation between the 36- and the 43-polyQ expressing cells *in vivo.* The frequency of formation of aggregates was stimulated by tTGase overexpression both in HA-36Q-GFP and HA-43Q-GFP transfected cells. Moreover treatment with cystamine, the specific inhibitor of tTGase, reduced tTGase activity of COS-7 cells and the percentage of aggregates both in HA-36Q-GFP and in HA-43Q-GFP transfected cells.

These results demonstrate a direct involvement of transglutaminase in aggregate formation. This could occur in neuronal cells of patients: larger polyQ stretches could favour cross-linking to susceptible lysine-containing proteins by transglutaminase reaction.

3. Turnover of aggregates. It has been shown that intracellular aggregates can be resolved by Hsp104 (40) and that polyglutamine residues co-localize in the proteasome with ubiquitin (40, 41). Moreover, a mutant version of hCdc34, a dominant inhibitor of ubiquitin-conjugating enzyme causes a decrease of the formation of aggregates (25). Our evidence indicates that inhibition of the proteasome ubiquitin-dependent degradation (35) increased the formation of aggregates in 43-polyQ protein expressing cells. Longer periods of incubation or higher doses of lactacystin did not induce formation of aggregates in 17-polyQ protein expressing cells. Taken together, these data suggest that ubiquitination is necessary for the formation of the aggregates and that proteasome activation is needed for their degradation.

It is possible that aggregates are not terminal metabolic products but are formed, dissolved or degraded in the normal cycle of the cells by Hsp104-like proteins or ubiquitin-dependent degradation. Unbalance between formation and degradation might result in the steady accumulation of intracellular aggregates.

We suggest that intracellular aggregates represent an intermediate and reversible step in the pathogenesis of CAG disease. Probably, the structure of the protein, transglutaminase activity, the concentration of chaperones might influence the net accumulation of the aggregates. Cells with high aggregate content accumulate them into the nuclei, are more sensitive to apoptotic stimuli and over time are more likely to die. The presence of aggregates probably reflects the ability of the cell to cope with an abnormal condition (unbalanced degradation or increased tTGase activity) triggered by the Q-expanded protein.

We do not exclude that a faster pathway can be activated bypassing the formation of visible aggregates and leading to cell death.

Our data indicate that an avenue for therapeutic intervention in neurodegenerative disorders will rely on the identification of genes and drugs that can stimulate the degradation pathway, or control neuronal TGase activity or reduce the expression of the Q-expanded protein.

ACKNOWLEDGMENTS

We thank Vittorio Gentile for the pSG5-tTGase expression vector and Laszlo Lorand for the antibody to guinea pig liver transglutaminase. This work was supported from “Murst-C.N.R. Biotechnology Program L.95/95”.

REFERENCES

1. The Huntington's Disease Collaborative Research Group (1993) *Cell* **26,** 72(6):971–983.
2. Nagafuchi, S., Yanagisawa, H., Sato, K., Shirayama, T., Ohsaki, E., Bundo, M., Takeda, T., Tadokoro, K., Kondo, I., and Murayama N. (1994) *Nat. Genet.* **6,** (1):14–18.
3. Koide, R., Ikeuchi, T., Onodera, O., Tanaka, H., Igarashi, S., Endo, K., Takahashi, H., Kondo, R., Ishikawa, A., and Hayashi, T. (1994) *Nat. Genet.* **6,** (1):9–13.
4. La Spada, A. R., Wilson, E. M., Lubahn, D. B., Harding, A. E., and Fischbeck, K. H. (1991) *Nature* **4,** 352(6330):77–79.
5. Nakamura, M., Mita, S., Murakami, T., Uchino, M., Watanabe, S., Tokunaga, M., Kumamoto, T., and Ando, M. (1994) *J. Neurol. Sci.* **122,** (1):74–79.
6. Banfi, S., Servadio, A., Chun., M. Y., Kwiatkowski, T. J., Jr., McCall, A. E., Duvick, L. A., Shen, Y., Roth, E. J., Orr, H. T., and Zoghbi, H. Y. (1994) *Nat. Genet.* **7,** (4):513–520.
7. Orr, H. T., Chung, M. Y., Banfi, S., Kwiatkowski, T. J., Jr., Servadio, A., Beaudet, A. L., McCall, A. E., Duvick, L. A., Ranum, L. P., and Zoghbi, H. Y. *Nat. Genet.* **4,** (3):221–226.
8. Pulst, S. M., Nechiporuk, A., Nechiporuk, T., Gispert, S., Chen, X. N., Lopes-Cendes, I., Pearlman, S., Starkman, S., Orozco-Diaz, G., Lunkes, A., DeJong, P., Rouleau, G. A., Auburger, G., Korenberg, J. R., Figueroa, C., and Sahba, S. (1996) *Nat. Genet.* **14,** (3):269–276.
9. Imbert, G., Saudou, F., Yvert, G., Devys, D., Trottier, Y., Garnier, J. M., Weber, C., Mandel, J. L., Cancel, G., Abbas, N., Durr, A., Didierjean, O., Stevanin, G., Agid, Y., and Brice, A. (1996) *Nat. Genet.* **14,** (3):285–291.
10. Kawaguchi, Y., Oamoto, T., Taniwaki, M., Aizawa, M., Inoue, M.,

Katayama, S., Kawakami, H., Nakamura, S., Nishimura, M., and Akiguchi, I. (1994) *Nat. Genet.* **8,** (3):221–228.

11. David, G., Abbas, N., Stevanin, G., Durr, A., Yvert, G., Cancel, G., Weber, C., Imbert G., Saudou, F., Antoniou, E., Drabkin, H., Gemmill, R., Giunti, P., Benomar, A., Wood, N., Ruberg, M., Agid, Y., Mandel, J. L., and Brice, A. (1997) *Nat. Genet.* **17,** (1):65–70.
12. Housman, D. (1995) *Nat. Genet.* **10,** (1):3–4.
13. Ross, C. A. (1997) *Neuron* **19,** (6):1147–1150.
14. Davies, S. W., Beardsall, K., Turmaine, M., DiFiglia, M., Aronin, N., and Bates, G. P. (1998) *Lancet* **10,** 351(9096):131–133.
15. Davies, S. W., Turmaine, M., Cozens, B. A., DiFiglia, M., Sharp, A. H., Ross, C. A., Scherzinger, E., Wanker, E. E., Mangiarini, L., and Bates, G. P. (1997) *Cell* **8,** 90(3):537–548.
16. Klement, I. A., Skinner, P. J., Kaytor, M. D., Yi, H., Hersch, S. M., Clark, H .B., Zoghbi, H. Y., and Orr, H. T. (1998) *Cell* **2,** 95(1):41–53.
17. DiFiglia, M., Sapp, E., Chase, K. O., Davies, S. W., Bates, G. P., Vonsattel, J. P., and Aronin, N. (1997) *Science* **26,** 277:1990–1993.
18. Skinner, P. J., Koshy, B. T., Cummings, C. J., Klement, I. A., Helin, K., Servadio, A., Zoghbi, H. Y., and Orr, H. T. (1997) *Nature* **30,** 389(6654):971–974.
19. Paulson, H. L., Perez, M. K., Trottier, Y., Trojanowski, J. Q., Subramony, S. H., Das, S. S., Vig, P., Mandel, J. L., Fischbeck, K. H., and Pittman, R. N. (1997) *Neuron* **19,** (2):333–344.
20. Holmberg, M., Duyckaerts, C., Durr, A., Cancel, G., Gourfinkel-An, I., Damier, P., Faucheux, B., Trottier, Y., Hirsch, E. C., Agid, Y., and Brice, A. (1998) *Hum. Mol. Genet.* **7,** (5):913–918.
21. Igarashi, S., Koide, R., Shimohata, T., Yamada, M., Hayashi, Y., Takano, H., Date, H., Oyake, M., Sato, T., Sato, A., Egawa, S., Ikeuchi, T., Tanaka, H., Nakano, R., Tanaka, K., Hozumi, I., Inuzuka, T., Takahashi, H., and Tsuji, S. (1998) *Nat. Genet.* **18,** (2):111–117.
22. Hackam, A. S., Singaraja, R., Wellington, C. L., Metzler, M., McCutcheon, K., Zhang, T., Kalchman, M., and Hayden, M. R. (1998) *J. Cell. Biol.* **1,** 141(5):1097–1105.
23. Ikeda, H., Yamaguchi, M., Sugai, S., Aze, Y., Narumiya, S., and Kakizuka, A. (1996) *Nat. Genet.* **13,** (2):196–202.
24. Sisodia, S. S. (1998) *Cell* **2,** 95(1):1–4.
25. Saudou, F., Finkbeiner, S., Devys, D., and Greenberg, M. E. (1998) *Cell* **2,** 95(1):55–66.
26. Cariello, L., de Cristofaro, T., Zanetti, L., Cuomo, T., Di Maio, L., Campanella, G., Rinaldi, S., Zanetti, P., Di Lauro, R., and Varrone, S. (1996) *Hum. Genet.* **98,** (6):633–635.
27. Cooper, A. J. L., Sheu, K. R., Burke, J. R., Onodera, O., Strittmatter, W. J., Roses, A. D., and Blass, J. P. (1997) *Proc. Natl. Acad. Sci.* **11,** 94(23):12604–12609.
28. Kahlem, P., Green, H., and Djian. P. (1998) *Mol. Cell.* **1,** (4):595–601.
29. Gentile, V., Sepe, C., Calvani, M., Melone, M. A., Cotrufo, R., Cooper, A. J., Blass, A. J. P., and Peluso, G. (1998) *Archours Biochem. Biophys.* **15,** 352(2):314–321.
30. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: a Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
31. Pages, G., Brunet, A., L'Allemain, G., and Pouyssegur, J. (1994) *EMBO J.* **13,** (13):3003–3310.
32. Wilson, I. A., Niman, H. L., Houghten, R. A., Cherenson, A. R., Connolly, M. L., and Lerner, R. A. (1984) *Cell* **37,** (3):767–778.
33. Lorand, L., and Conrad, M. (1984) *Mol. Cell. Biochem.* **58,** 9–35.
34. Siefring, G. E., Jr., Apostol, A. B., Velasco, P. T, and Lorand, L. (1978) *Biochemistry* **27,** 17(13):2598–2604.
35. Oda, K., Ikehara, Y., and Omura, S. (1996) *Biochem. Biophys. Res. Commun.* **219**(3), 800–805.
36. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254.
37. Lorand, L., Campbell-WilKes, L. K., and Cooperstein, L. (1972) *Anal. Biochem.* **50,** 623–631.
38. Kahlem, P., Terre, C., Green, H., and Djian, P. (1996) *Proc. Natl. Acad. Sci.* **10,** 93(25):14580–14585.
39. Cooper, A. J., Sheu, K. F., Burke, J. R., Onodera, O., Strittmatter, W. J., Roses, A. D., and Blass, J. P. (1997) *J. Neurochem.* **69,** (1):431–434.
40. Cummings, C. J., Mancini, M. A., Antalffy, B., DeFranco, D. B., Orr, H. T., and Zoghbi, H. Y. (1998) *Nat. Genet.* **19,** (2):148–154.
41. Kalchman, M. A., Graham, R. K., Xia, G., Koide, H. B., Hodgson, J. G., Graham, K. C., Goldberg, Y. P., Gietz, R. D., Pickart, C. M., and Hayden, M. R. (1996) *J. Biol. Chem.* **9,** 271(32):19385–19394.