Abstract. The cause of Huntington’s disease (HD) is a pathological expansion of the polyglutamine domain within the NH2-terminal region of huntingtin. Neuronal intranuclear inclusions and cytoplasmic aggregates composed of the mutant huntingtin within certain neuronal populations are a characteristic hallmark of HD. Because in vitro expanded polyglutamine repeats are glutaminyl-donor substrates of tissue transglutaminase (tTG), it has been hypothesized that tTG may contribute to the formation of these aggregates in HD. Therefore, it is of fundamental importance to establish whether tTG plays a significant role in the formation of mutant huntingtin aggregates in the cell. Human neuroblastoma SH-SY5Y cells were stably transfected with truncated NH2-terminal huntingtin constructs containing 18 (wild type) or 82 (mutant) glutamines. In the cells expressing the mutant truncated huntingtin construct, numerous SDS-resistant aggregates were present in the cytoplasm and nucleus. Even though numerous aggregates were present in the mutant huntingtin-expressing cells, tTG did not coprecipitate with mutant truncated huntingtin. Further, tTG was totally excluded from the aggregates, and significantly increasing tTG expression had no effect on the number of aggregates or their intracellular localization (cytoplasm or nucleus). When a YFP-tagged mutant truncated huntingtin construct was transiently transfected into cells that express no detectable tTG due to stable transfection with a tTG antisense construct, there was extensive aggregate formation. These findings clearly demonstrate that tTG is not required for aggregate formation, and does not facilitate the process of aggregate formation. Therefore, in HD, as well as in other polyglutamine diseases, tTG is unlikely to play a role in the formation of aggregates.

Key words: Huntington’s disease • inclusions • polyglutamine • polar zipper • isopeptide bond

Introduction

Huntington’s disease (HD)\(^1\) is an autosomal-dominant neurodegenerative disorder caused by pathological expansion of polyglutamine repeats in the NH2-terminal region of a 350-kD protein of unknown function called huntingtin (The Huntington’s Disease Collaborative Research Group, 1993). Although mutant huntingtin is a fundamental cause of HD, the specific molecular mechanisms responsible for the selective neuronal degeneration remain to be elucidated. A pathological hallmark of HD brain is the presence of cytoplasmic and nuclear aggregates in specific neuronal populations that contain the NH2-terminal region of mutant huntingtin (DiFiglia et al., 1997). Further, protein aggregates have been reported in the brains of transgenic HD mouse models, as well as in transfected cell models of HD (Davies et al., 1997; Saudou et al., 1998; Persichetti et al., 1999; Yamamoto et al., 2000). Although it is clear that cytoplasmic and nuclear inclusions occur in HD, and in other polyglutamine diseases, the role of the inclusions in the pathogenesis of the disease remains inconclusive. Indeed, there have been reports indicating that aggregates may be beneficial (Klement et al., 1998; Saudou et al., 1998; Gutekunst et al., 1999), while others studies have concluded that the aggregates are likely to be toxic (Hackam et al., 1998; Hackam et al., 1999; Li, 2000).

Two mechanisms have been proposed to explain how expanded polyglutamine domains form insoluble aggregates. It has been hypothesized that the expanded polyglutamine repeats may interact with each other through a polar zipper and thus contribute to aggregate formation (Perutz et al., 1994). Further, it has been hypothesized that tissue transglutaminase (tTG), perhaps in conjunction with the polar zipper mechanism, may be a contributing factor in the formation of these aggregates (Cooper et al., 1997, 1999; Kahlem et al., 1996, 1998). Because it has been proposed...
that tTG may be a potential therapeutic target in the treat-
ment of polyglutamine diseases (Igarashi et al., 1998), it is
of critical importance to determine the contribution of
tTG to the formation of inclusions.

The transglutaminases are a family of calcium-depend-
ent enzymes that catalyze the formation of \( \varepsilon-(\gamma\text{-glu-
tamyl)lysine isopeptide bonds between substrate proteins,}
rendering the resulting cross-linked protein complexes
insoluble (Folk, 1983; Lorand and Conrad, 1984; Green-
berg et al., 1991). Transglutaminases also catalyze the in-
corporation of polynamines into substrate proteins (Lorand
and Conrad, 1984; Greenberg et al., 1991). Because the
peptide-bound glutamine is the primary determining
factor for a transglutaminase-catalyzed reaction, it has
been hypothesized that increasing the number of glu-
tamines in a protein beyond a certain threshold may result
in the protein becoming a transglutaminase substrate
(Green, 1993). tTG is found within neurons (Miller and
Anderton, 1986; Appelt et al., 1996; Lesort et al., 1999)
and is increased in specific areas affected in HD brain
(Karpjuk et al., 1999; Lesort et al., 1999). Further, the in-
crease in tTG expression in HD brain occurred within
neurons (Lesort et al., 1999). Previously, it had been
shown that tTG levels in SH-SY5Y cells are significantly
increased by treatment with retinoic acid, and further tTG
can be activated by increasing intracellular calcium levels
(Zhang et al., 1998). Even though it has been demonstrated
that polyglutamine repeat domains (Kahlem et al., 1996;
Cooper et al., 1997) and mutant huntingtin (Kahlem et
al., 1998) are substrates for tTG in vitro, it has not yet
been shown that huntingtin interacts with or is modified
by tTG in situ. To determine the potential role of tTG in
aggregate formation, SH-SY5Y cell lines stably expressing
mutant or wild-type truncated NH\(_2\)-terminal huntingtin
constructs were established. Using these cells, we demon-
strate that tTG and the mutant truncated huntingtin do
not interact, and further huntingtin is not modified by tTG
in situ. Moreover, immunocytochemical analysis revealed
that tTG was totally excluded from the aggregates that
form in the cells expressing the mutant huntingtin con-
struct. Finally, transient transfection of the YFP-tagged
mutant huntingtin construct into cells that do not express
detectable levels of tTG due to stable transfection with an
antisense tTG construct, resulted in significant aggregate
formation. In addition, the formation of mutant huntingtin
aggregates was equivalent in tTG antisense cells and in
cells stably transfected with vector only. These data clearly
prove that tTG is unlikely to be a contributing fac-
tor to the formation of aggregates in HD brain.

**Materials and Methods**

**Construction of Expression Plasmids**

Expression constructs of truncated huntingtin with 63 amino acids,
pcDNA3.1-N63-18Q-Myc/His, were created by subcloning a fragment of
the huntingtin cDNA (bp 314–505) generated by PCR and included BamHI
and XhoI restriction sites. The 189-bp product of interest was digested with
BamHI and XhoI, and then subcloned into pcDNA3.1/His A vector (Coo-
per et al., 1998). The BamHI-XhoI huntingtin cDNA fragments were also
subcloned into the Amersham Pharmacia Biotech and XhoI sites of the
pECFP-N1 vector (CLONTECH Laboratories, Inc. (N-Q18) (pECFP-N1-
18Q) or into the NheI and XhoI sites of the pYEFP-N1 vector (CLON-
TECH Laboratories, Inc. (N-Q82) (pYEFP-N1-82Q). The BamHI and
NheI restriction sites were filled in with T4 DNA polymerase before liga-
tion. All DNA constructs were verified by automated sequencing.

**Cell Human neuroblastoma SH-SY5Y cells were transfected by electropora-
tion (Gene Pulser II; Bio-Rad Laboratories) according to the supplier’s
instructions. SH-SY5Y cells stably expressing pcDNA3.1 vector alone,
N-Q82 (wild-type truncated huntingtin) or N-Q18 (mutant truncated hun-
tingtin) were selected based on their resistance to G418, subcloned, and
maintained on Corning dishes in RPMI 1640 medium supplemented with
20 mM glutamine, 10 U/ml penicillin, 100 \( \mu \text{g/ml} \) streptomycin, 5% fetal
clonal II serum, 10% horse serum, and 100 \( \mu \text{g/ml} \) G418 (GIBCO BRL). To
differentiate the cells, the cells were grown in medium containing 1% fetal
clonal II serum, 5% horse serum, and 20 \( \mu \text{M} \) retinoic acid for 5 d. Previous
studies have shown that treatment of SH-SY5Y cells with retinoic acid re-
results in a significant increase in tTG expression (Zhang et al., 1998). Ex-
cept where indicated, all studies were carried out on cells that were
treated with retinoic acid and therefore express high levels of tTG (Zhang
et al., 1998). All experiments were carried out on sub-confluent cultures.

**Cell Viability Assay**

To determine whether truncated mutant huntingtin decreases basal cell
viability, LDH release was measured in the cell lines (Decker and Loh-
mann-Matthes, 1988; Davis et al., 1997). There was no significant difference
among groups, indicating no loss of cell viability due to the expression of
the mutant huntingtin (data not shown). Further, there was no evidence of
increased apoptosis in the mutant huntingtin-expressing cells as deter-
mined by the presence of condensed chromatin as detected by Hoechst
staining (data not shown).

**Antibodies**

A rabbit polyclonal huntingtin antibody was prepared using a synthetic
peptide composed of the first 17 amino acids of huntingtin protein and af-
finity purified using the antigen (a gift from Dr. P. Detloff, University of
Alabama at Birmingham, Birmingham, AL) (Lin et al., 2001). This hun-
tingtin antibody was made and purified by Research Genetics. The anti-
gen and reactivity of this NH\(_2\)-terminal huntingtin antibody are identical
to the NH\(_2\)-terminal huntingtin antibody described by Sawa et al. (1999).
Mouse monoclonal antibodies to C-myc (Zymed Laboratories), tTG
(TG100 and CUB7402) (Neomarkers), Hsp70 (StressGen Biotechnolo-
gies), and ubiquitin (Zymed Laboratories) were also used in this study.

**Immunoblotting**

To evaluate the expression levels of tTG and the huntingtin proteins in
naive and differentiated cells, extracts from cells were prepared and quan-
titatively immunoblotted. Cells were harvested in cold PBS, collected by
centrifugation, resuspended in a homogenizing buffer (50 mM Tris-HCl,
PH 7.5, 150 mM NaCl, 1 mM EDTA, 0.5% NP-40, 0.1 mM phenylmethyl-
sulfonyl fluoride, and a 10 \( \mu \text{g/ml} \) concentration each of aprotinin, leupep-
tin, and pepstatin), and sonicated on ice. Protein concentrations of the ho-
genates were determined using the BCA method (Pierce Chemical Co.) and
diluted to a final concentration of 2 mg/ml with 2\( \times \) reducing stop
buffer (0.25 M Tris-HCl, pH 6.8, 5 mM EDTA, 5 mM EGTA, 25 mM diothio-
reitol, 2% SDS, 10% glycerol, and bromophenol blue as the track-
dye). Samples (30 \( \mu \text{g} \) of protein) were resolved on 4–20% gradient
SDS-polyacrylamide gels, and transferred to nitrocellulose. Blots were
blocked in 5% nonfat dry milk in TBST (20 mM Tris–HCl, pH 7.6, 137
mM NaCl, 0.05% Tween 20) for 1 h at room temperature. The blots were
then incubated with the anti–tTG monoclonal antibody TG100 (1:750;
Neomarkers) or with a polyclonal huntingtin antibody (1:20,000) in the
same buffer overnight at 4°C. The membranes were then washed three
times with TBST and incubated with HRP-conjugated goat anti–mouse
IgG (1:2,000) for tTG, or with HRP-conjugated goat anti–rabbit IgG
(1:2,000) for the polyclonal huntingtin antibody for 2 h at room tempera-
ture. The membranes were rinsed three times for 30 min with TBST, fol-
dowed by four quick rinses with distilled water, and developed with the en-
hanced chemiluminescence method (Amersham Pharmacia Biotech).

**Coimmunoprecipitation**

Cell lysates were prepared in homogenizing buffer and samples containing
200 \( \mu \text{g} \) of protein were precleared for 1 h at 4°C with protein A-Sepharose
(Amersham Pharmacia Biotech) before immunoprecipitation of hunting-
tin. Precleared samples were immunoprecipitated overnight at 4°C with
the polyclonal NH\(_2\)-terminal huntingtin antibody. To control for nonspec-
ific binding, some samples were immunoprecipitated with nonimmune
rabbit IgG. Protein A-Sepharose was added, and the incubation contin-
Immunocytochemistry

Cells were seeded on poly-D-lysine-coated cover slips in 24-well plates. 24 h later, cells were fixed in 90% methanol, 50 mM EGTA, pH 6.0, for 5 min at −20°C (Melan and Shuler, 1992), incubated for 10 min with 0.2% Triton X-100 in PBS, and rinsed three times with PBS, before incubation with 5% bovine serum albumin in PBS for 90 min to reduce the background. Cells were then incubated at room temperature for 90 min with the polyclonal NH₂-terminal huntingtin antibody (1:20,000) in 5% BSA/PBS. Cells were then rinsed three times with PBS, incubated for 60 min at room temperature with FITC-conjugated anti-rabbit IgG (1:200) in 5% BSA/PBS. For colocalization studies, cells were treated with 20 μM retinoic acid for 5 d before fixation and permeabilization. Cells were then incubated at room temperature for 90 min with the polyclonal NH₂-terminal huntingtin antibody (1:20,000) and the monoclonal rTG antibody CUB7402 (1:20), or the monoclonal Hsp70 antibody (1:200), or the monoclonal ubiquitin antibody (1:200). Cells were then rinsed three times with PBS and incubated for 60 min at room temperature with FITC-conjugated anti-rabbit IgG (1:200) and Texas red-conjugated anti-mouse IgG (1:100). Cells were then rinsed with PBS and incubated with 5 μg/ml Hoescht at 30 min at room temperature. Coverslips were then washed extensively in PBS and mounted. Cells were viewed using confocal microscopy and images were acquired by sequential scanning at the appropriate wavelengths. The digitally stored images were combined and displayed with the accompanying software and Adobe Photoshop 4.0.

Expression of C/YFP-tagged Huntingtin Constructs in Antisense rTG Cells

To essentially abolish rTG expression, SH-SY5Y cells were stably transfected with an antisense rTG construct as described previously (Tucholski et al., 2001). There is no detectable rTG expression or transglutaminase activity in these antisense rTG cells (Tucholski et al., 2001).

Results

To demonstrate the specificity of the polyclonal NH₂-terminal huntingtin antibody, cell extracts from the vector control cells were immunoblotted. The immunoblot in Fig. 1 A demonstrates that the antibody recognizes en-
Endogenous huntingtin. However, the levels of endogenous huntingtin in these cells is low, and this immunoblot had to be exposed a relatively long time to bring up the signal. In Fig. 1B immunocytochemical analyses with the polyclonal NH2-terminal huntingtin antibody in vector-transfected and N-Q18 cells are shown. These data demonstrate that immunoreactivity is extremely low in the vector cells; however, in the N-Q18 cells, expression is significantly elevated.

To determine the putative role of tTG in aggregate formation, SH-SY5Y cells stably transfected with the truncated NH2-terminal huntingtin constructs N-Q18 (wild-type truncated huntingtin) or N-Q82 (mutant truncated huntingtin) were established. Immunoblot analysis revealed that in the N-Q18 cells there was a huntingtin immunoreactive band, as expected, at ~14 kD (Fig. 2). In the N-Q82 cells, huntingtin immunoreactive bands were observed at ~23 and 55 kD. The 55-kD band is likely to be a complex containing the truncated NH2-terminal huntingtin. Treatment of the cells with RA resulted in a significant increase in tTG levels, but did not alter the expression or levels of the truncated NH2-terminal huntingtin protein.

Figure 2. Representative immunoblots of the expression of truncated NH2-terminal huntingtin (top) and tTG (bottom) in SH-SY5Y cells stably expressing truncated NH2-terminal huntingtin constructs, N-Q18 or N-Q82. Cells were incubated in the absence (−) or presence (+) of 20 μM retinoic acid (RA) for 5 d before immunoblotting for huntingtin or tTG. N-Q18 cells displayed a major huntingtin immunoreactive band at ~14 kD and in N-Q82 cells, two truncated NH2-terminal huntingtin immunoreactive bands were detected at ~23 and 55 kD. The 55-kD band is likely to be a complex containing the truncated NH2-terminal huntingtin. Treatment of the cells with RA resulted in a significant increase in tTG levels, but did not alter the expression or levels of the truncated NH2-terminal huntingtin protein.

Figure 3. Huntingtin immunostaining in cells stably transfected with N-Q18 or N-Q82. Huntingtin localization was detected with an NH2-terminal polyclonal huntingtin antibody. In the N-Q18 cells, huntingtin immunostaining was diffuse throughout the cytoplasm (a). In contrast, in the N-Q82 cells, huntingtin formed large aggregates in the cytoplasm (b and c) and also in the nucleus (b and d). Note also the presence of diffuse huntingtin immunoreactivity in the nucleus of N-Q82 cells (d).
To evaluate the intracellular distribution of N-Q18 and N-Q82, immunocytochemical analyses were carried out using the polyclonal NH$_2$-terminal huntingtin antibody. In N-Q18 cells huntingtin immunoreactivity was diffuse through the cytoplasm in all the cells (Fig. 3 a). In the N-Q82 cells, diffuse huntingtin immunoreactivity was also observed; however, in ~13% of the cells cytoplasmic and/or nuclear aggregates were observed (Fig. 3, b–d). There was also evidence of diffuse huntingtin staining in the nucleus of N-Q82 cells (Fig. 3 d). Identical results were obtained with the monoclonal antibody to c-myc (data not shown).

To further evaluate the inclusions formed by N-Q82, cells were costained for huntingtin and ubiquitin or Hsp70, as inclusions in models of HD and other polyglutamine diseases are usually ubiquitinated and often reactive with antibodies to heat shock proteins (Davies et al., 1997; Saudou et al., 1998; Wyttenbach et al., 2000). The majority of the aggregates were immunopositive for both ubiquitin and hsp70 (Fig. 4). Interestingly, both the huntingtin and ubiquitin antibodies stained the peripheral areas of the inclusions, while the Hsp70 antibody stained the entire inclusion (Fig. 4). The reason for the peripheral staining pattern of the aggregates with the NH$_2$-terminal huntingtin and ubiquitin antibodies is unknown, but may be due to the organization of the inclusion that results in “masking” of the NH$_2$-terminal portion of the huntingtin at the center of the inclusion. In addition, when N-Q82 cells were costained for huntingtin and C-myc, there was complete overlap of the immunostaining, clearly demonstrating that the truncated huntingtin is a primary constituent of the aggregates (Fig. 5).

To determine whether tTG and highly truncated huntingtin interact, coimmunoprecipitation assays were carried out. N-Q18 huntingtin coprecipitated with tTG; however, this interaction was not increased when tTG was

![Figure 4](image_url)

**Figure 4.** Aggregates of N-Q82 are ubiquitinated and colocalize with Hsp70. Cells were double labeled with the polyclonal huntingtin antibody (green) and with the monoclonal ubiquitin or Hsp70 antibody (red), and nuclei were counterstained with Hoechst (blue). Aggregates of N-Q82 were ubiquitinated and ubiquitin immunoreactivity colocalized in the peripheral areas of the inclusion (a–c). Hsp70 immunoreactivity colocalized with the aggregates and stained the entire inclusion (d–f).

![Figure 5](image_url)

**Figure 5.** Colocalization of huntingtin and C-myc immunoreactivity in N-Q82 cells. N-Q82 cells were double labeled with the polyclonal huntingtin antibody (green) and with the monoclonal C-myc antibody (red). Nuclei were counterstained with Hoechst (blue). Huntingtin and C-myc immunoreactivity showed complete overlap, especially at the levels of the aggregates.
activated (Fig. 6 A). tTG is associating with the N-Q18, as when endogenous full-length huntingtin was immunoprecipitated from the parental SH-SY5Y cells line, tTG did not coimmunoprecipitate with the huntingtin (data not shown). In contrast, N-Q82 huntingtin only showed a very weak interaction with tTG (Fig. 6 A). SDS-resistant material that contained the N-Q82 huntingtin was detected at the top of the gel, indicating that insoluble aggregates had formed. When the N-Q82 or N-Q18 cells were immunoprecipitated with nonimmune rabbit IgG, no huntingtin precipitated (Fig. 6 B). To determine whether tTG colocalized with the highly truncated huntingtin, costaining was carried out with the huntingtin and tTG antibodies. In the N-Q18 cells, both huntingtin and tTG immunoreactivity were diffuse through the cytoplasm (Fig. 7, a–c). In the N-Q82 cells, there was also no specific colocalization of tTG and huntingtin and, further, tTG immunoreactivity was completely excluded from the aggregates (Fig. 7, d–i). In addition, the number of N-Q82 cells with aggregates was similar in control conditions and after increased tTG expression due to retinoic acid treatment (Fig. 8). To determine whether tTG modifies highly truncated huntingtin or associated proteins, cells were prelabeled with 5-(biotinamido)pentylamine, incubated in the absence or presence of maitotoxin, and immunoprecipitated with the polyclonal huntingtin antibody, probed with a C-myc antibody, and then stripped and reprobed with neutravidin-HRP. The results of these experiments demonstrated that neither truncated huntingtin nor any huntingtin-associated proteins were modified by tTG (data not shown).

To confirm that tTG does not play a role in the formation of mutant huntingtin inclusions, pEYFP-N1-82Q or pCYFP-N1-18Q were transiently transfected into cells stably transfected with vector (pcDNA3.1) only or into cells stably transfected with an antisense tTG construct. In the antisense tTG cells, tTG is undetectable and there is no measurable transglutaminase activity (Tucholski et al., 2001). As expected, transient transfection of pCYFP-N1-18Q into either the pcDNA or antisense tTG cells did not result in any aggregate formation (data not shown). In contrast, transient transfection of pEYFP-N1-82Q resulted in abundant aggregate formation in both the pcDNA and antisense cells (Fig. 9). These studies clearly demonstrate that it is unlikely that tTG plays a role in the formation of inclusions in HD brain.

**Discussion**

The hypothesis that tTG contributes to the etiology of HD was first proposed by Green (1993) before the identification of huntingtin aggregates in HD brain. With the discovery of intranuclear and cytoplasmic inclusions of mutant huntingtin in HD brain (DiFiglia et al., 1997) and in transgenic mouse models for HD (Davies et al., 1997), it was further hypothesized that tTG may facilitate the formation of these aggregates by selectively cross linking mutant huntingtin (Cooper et al., 1999). Indeed, it has even been suggested that tTG may be a potential therapeutic target in the treatment of polyglutamine diseases (Igarashi et al., 1998). Additionally, tTG levels and transglutaminase activity are significantly increased in HD brain (Karpuj et al., 1999; Lesort et al., 1999) in a grade- and region-dependent manner, and there is a significant increase in the tTG immunoreactivity in specific neuronal populations in HD brain (Lesort et al., 1999). Although these findings are intriguing, there has been no direct demon-
stration that tTG is involved in the formation of aggregates in HD or other polyglutamine diseases. Therefore, the goals of this study were to determine whether or not tTG and huntingtin interact in situ, and whether tTG facilitates the formation of mutant huntingtin aggregates.

It has been well documented that in vitro polyglutamine constructs and mutant huntingtin are substrates for tTG (Kahlem et al., 1996, 1998; Cooper et al., 1997). However, the ability of tTG to modify mutant huntingtin in situ has not been demonstrated, and the putative contribution of tTG in the formation of polyglutamine aggregates is still controversial. It was reported that transglutaminase inhibitors suppressed aggregate formation and apoptosis in cells expressing truncated DRPLA protein with an expanded polyglutamine domain (Igarashi et al., 1998). These findings need to be interpreted with some caution as both transglutaminase inhibitors used in this study can also inhibit other enzymes (e.g., caspases), and in some cases the transglutaminase inhibitors reduced apoptotic cell death, but were ineffective in blocking the formation of aggregates (Lorand, 1998). In another study, tTG overexpression was reported to increase the aggregate formation of synthetic fusion proteins containing 36 or 43 glutamines. However, the percent increase in aggregate formation induced by tTG overexpression was only ~10–15% (de Cristofaro et al., 1999). These authors also presented data that cystamine, a transglutaminase inhibitor, at relatively high concentrations (0.25 and 0.50 mM) reduced aggregate formation (de Cristofaro et al., 1999). In another study, the same inhibitor (0.20 mM) had no effect in mutant huntingtin aggregate formation, and, when the inhibitor was used at higher concentrations (0.5 mM), it was cytotoxic (Kim et al., 1999). These findings indicate the need for further investigations before a reasonable case can be made for the involvement of tTG in the etiology of HD.

In the present study, we used human neuroblastoma SH-SY5Y cells stably transfectsed with wild-type and mutant truncated huntingtin to investigate the role of tTG in huntingtin modification and aggregate formation. SH-SY5Y

Figure 7. Truncated NH2-terminal huntingtin and tTG do not colocalize. Cells were double labeled with the polyclonal huntingtin antibody (green) and with the monoclonal tTG antibody CUB7402 (red); nuclei were counterstained with Hoechst (blue). In cells stably expressing N-Q18, huntingtin (a) and tTG immunoreactivities (b) were diffuse throughout the cytoplasm and did not show specific colocalization (c). In contrast, cells stably expressing N-Q82 showed large huntingtin immunoreactive aggregates in the cytoplasm (d and e) and in the nucleus (g). In these cells, tTG immunoreactivity was also diffuse throughout the cytoplasm and, remarkably, appeared completely excluded from the huntingtin aggregates (e, f, h, and i). These results demonstrate that the huntingtin aggregates and tTG do not colocalize.
cells are an excellent model system for these studies because they have neuronal-like features (Preis et al., 1988; Pahlman et al., 1990; Jalava et al., 1992), and tTG expression is robustly upregulated in response to retinoic acid (Zhang et al., 1998). Therefore, alterations in huntingtin can be evaluated in conditions when tTG levels are low, and also when the levels and activity of tTG are elevated. In these cells, N-Q82 did not increase cell death under basal conditions. However, it should be noted that overexpression of N-Q82 does sensitize the cells to apoptotic stressors (Chun, W., M. Lesort, and G.V.W. Johnson, unpublished observations, manuscript in preparation). This is in agreement with a previous study reporting that transient transfection of the N-Q82 construct into neuroblastoma N2a cells resulted in aggregate formation and increased sensitivity to staurosporine-induced apoptosis; however, no changes in cell viability under basal conditions were reported (Cooper et al., 1998). Similar findings were reported when mutant truncated huntingtin constructs were transiently transfected into cells that had been stably transfected with antisense tTG, and therefore express undetectable levels of the protein, insoluble aggregates were still formed. Further, tTG is virtually undetectable in mouse (C57BL) brain by immunoblot analysis (Lesort, M., unpublished observations), although inclusions are usually found in the brains of mouse models of HD (Davies et al., 1997; Schilling et al., 1999; Wheeler et al., 2000). Indeed, given the fact that the cross-linking and polyamination reactions catalyzed by tTG are competing reactions (Lorand and Conrad, 1984; Greenberg et al., 1991), and the fact that the levels of polyamines in the brain are in the millimolar range (Morrison et al., 1995), it seems unlikely that tTG catalyzes cross-linking reactions within the cells. Therefore, another mechanism, such as the formation of β-sheets via the glutamine repeats acting as polar zippers (Perutz et al., 1994; Stott et al., 1995), is more likely to be responsible for the formation of huntingtin aggregates in HD brain. In vitro, polyglutamine constructs form β-sheets that are held together by hydrogen bonds (Perutz et al., 1994; Stott et al., 1995; Scherzinger et al., 1997). Congo red selectively stains β-sheet structure, and aggregates from HD brain (Huang et al., 1998), as well as aggregates between tTG and the mutant truncated huntingtin. In all cases, no in situ modification of huntingtin tTG was observed. This demonstrates that although huntingtin is an in vitro substrate of tTG (Kahlem et al., 1998), it is unlikely to be modified by tTG in vivo. Furthermore, we found that tTG was totally excluded from the insoluble huntingtin aggregates in the N-Q82 cells, although other proteins such as Hsp70 showed a strong colocalization with huntingtin at the level of the aggregates. In addition, the number and size of the aggregates in the N-Q82 cells expressing either low or high levels (due to retinoic acid treatment) of tTG were not significantly different. These findings demonstrate that it is unlikely that tTG plays a role in aggregate formation in HD brain. Indeed, the data are expressed as mean% ± SEM.
formed from mutant huntingtin in vitro (Scherzinger et al., 1997), stain with Congo red. These data suggest that hydro- gen bonds between the side chain amides of glutamine and the amides of the polypeptide backbone may be the essential process in huntingtin aggregate formation in HD (Perutz, 1994; Perutz et al., 1994).

It should be noted that the appearance of the aggregates in the cells stably expressing N-Q82 compared with those in which the N-Q82 construct was transiently transfected was significantly different. In the transient transfection model, the protein is expressed rapidly and at very high levels, which likely results in the more amorphous appearance of the aggregates, as has been shown by others (Hackam et al., 1998). In the stable cells, expression of N-Q82 resulted in the formation of very well-defined aggregates, which is likely due to the fact that expression is more controlled in the stably transfected cells, and hence the proteins can organize into more well-defined structures (Lunkes and Mandel, 1998).

In conclusion, the results of the present study demonstrate that tTG associates with wild-type, but not mutant, truncated huntingtin; however, tTG does not modify the huntingtin, either wild-type or mutant. Further, tTG is totally excluded from the inclusions in the mutant truncated huntingtin-expressing cells, and mutant huntingtin aggregates form in the absence of tTG. These findings clearly demonstrate that tTG is not essential for the formation of huntingtin aggregates in HD brain.

The authors thank Dr. Peter Detloff for the generous gift of the polyclonal huntingtin antibody.

This work was supported by National Institutes of Health grant AG12396 (G.V.W. Johnson) and a fellowship from the Hereditary Disease Foundation (M. Lesort).

Submitted: 16 October 2000
Revised: 2 February 2001
Accepted: 6 February 2001

References

Appelt, D.M., G.C. Kopen, L.J. Boyne, and B.J. Balin. 1996. Localization of transglutaminase in hippocampal neuron: implications for Alzheimer’s disease. J. Histochem. Cytochem. 44:1421–1427.

Cooper, A.J., K.F. Sheu, J.R. Burke, O. Onodera, W.J. Strittmatter, A.H. Sharp, Z. Kaminsky, J.K. Cooper, J.K., G. Schilling, M.F. Peters, W.J. Herring, A.H. Sharp, Z. Kaminsky, M. Williams, P.H. Reddy, D. Tagle, et al. 1999. Mutant huntingtin expression in clonal striatal cells: dissociation on inclusion formation and neuronal selective vulnerability. J. Neurochem. 72:889–899.

Cooper, A.J., K.F. Sheu, J.R. Burke, W.J. Strittmatter, V. Mittal, J.P. Vonsattel, M.L. Kahlem, P., C. Terre, H. Green, and P. Dijan. 1998. Transglutaminase action imitates Huntington’s disease: selective polymerization of Huntingtin containing expanded polyglutamine. Mol. Cell. 1:595–601.

Kahlem, P., C. Terre, H. Green, and P. Dijan. 1996. Peptide containing glutamine repeats as substrates for transglutaminase-catalyzed cross-linking: relevance to diseases of the nervous system. Proc. Natl. Acad. Sci. USA. 93:14580–14585.

Karpuk, M.V., H. Garren, H. Shutt, D.L. Price, J. Gussella, M.W. Becher, and L. Steinmann. 1999. Transglutaminase aggregates huntingtin into nonamyloidogenic polymers, and its enzymatic activity increases in Huntington’s disease brain nuclei. Proc. Natl. Acad. Sci. USA. 96:7388–7393.

Kim, M., H.-S. Lee, G. LaForêt, C. McIntyre, J.M. Eileen, P. Chang, T.W. Kim, M. Williams, P.H. Reddy, D. Tagle, et al. 1999. Transglutaminase expression in clonal striatal cells: dissociation on inclusion formation and neuronal survival by caspase inhibition. J. Neurosci. 19:964–973.

Klement, I.A., P.J. Skinner, M.D. Kaytor, H. Yi, S.M. Hersch, H.B. Clark, H.Y. Zoogo, and H.T. Orr. 1998; Axatin-1 nuclear localization and aggregation, role in polyglutamine-induced disease in SCA1 transgenic mice. Cell. 95:41–53.

Lemkau, B., M. Chun, G.V. Johnson, and R.J. Ferrante. 1999. Tissue transglutaminase is increased in Huntington’s disease brain. J. Neurochem. 73:2018–2027.

Li, X.J. 2000. The early cellular pathology of Huntington’s disease. Mol. Neurobiol. 20:3–17.

Lin, C.-H., S. Talikosen-Greene, W.-M. Chein, J. Cearley, W. Jackson, A. Crouse, S. Ren, X.-J. Li, R. Albin, and P. Detloff. 2001. Neurological abnormalities in a knock-in mouse model of Huntington’s disease. Hum. Mol. Genet. 10:137–144.

Lorand, L. 1998. DRPLA aggregation and transglutaminase, revisited. Nat. Genet. 20:231.

Lorand, L., and L.S. Conrad. 1984. Transglutaminases. Mol. Cell. Biochem. 58:3–20.

Vanhoutte, A., and M. Mandel. 1998. A novel gene containing a trinucleotide repeat that is expanded and unstable on Huntington’s disease chromosomes. Cell. 71:971–983.

Igarashi, S., K. Koide, T. Shimohata, M. Yamada, Y. Hayashi, H. Takano, H. Daita, M. Oyake, T. Sato, A. Sato, et al. 1998. Suppression of aggregate formation and apoptosis by transglutaminase inhibitors in cells expressing truncated DRPLA protein with an expanded polyglutamine stretch. Nat. Genet. 18:111–117.

Jalava, A., J. Heikila, M. Lintunen, K. Akerman, and S. Pahlin. 1992. Stau- rorosipine induces a neuronal phenotype in SH-SY5Y human neuroblastoma cells that resembles that induced by the phorbol ester 12-O-tetradecanoyl phorbol-13-acetate (TPA). FEBS Lett. 300:114–118.

Kahlem, P., H. Green, and P. Dijan. 1998. Transglutaminase action imitates Huntington’s disease: selective polymerization of Huntingtin containing expanded polyglutamine. Mol. Cell. 1:595–601.

Lemkau, B., M. Chun, G.V. Johnson, and R.J. Ferrante. 1999. Tissue transglutaminase is increased in Huntington’s disease brain. J. Neurochem. 73:2018–2027.

Li, X.J. 2000. The early cellular pathology of Huntington’s disease. Mol. Neurobiol. 20:3–17.

Lin, C.-H., S. Talikosen-Greene, W.-M. Chein, J. Cearley, W. Jackson, A. Crouse, S. Ren, X.-J. Li, R. Albin, and P. Detloff. 2001. Neurological abnormalities in a knock-in mouse model of Huntington’s disease. Hum. Mol. Genet. 10:137–144.

Lorand, L. 1998. DRPLA aggregation and transglutaminase, revisited. Nat. Genet. 20:231.

Lorand, L., and L.S. Conrad. 1984. Transglutaminases. Mol. Cell. Biochem. 58:3–20.

Lunkes, A., and J. Mandel. 1998. A cellular model that recapitulates major pathogenic steps of Huntington’s disease. Hum. Mol. Genet. 7:1335–1361.

Melan, M.A., and G. Sluder. 1992. Redistribution and differential extraction of soluble proteins in permeabilized cultured cells. Implications for immunofluo- rescence microscopy. J. Cell Sci. 101:751–743.

Miller, C.C., and B.H. Anderton. 1986. Transglutaminase and the neuronal cytoskeleton in Alzheimer’s disease. J. Neurochem. 46:1912–1922.

Morrison, L.D., L. Becker, L.C. Ang, and S.J. Kish. 1995. Polyamines in human brain: regional distribution and influence of aging. J. Neurochem. 68:1335–1361.

N. Aoron. 1997. Aggregation of huntingtin in neuronal intranuclear inclu- sions and dystrophic neurites. Proc. Natl. Acad. Sci. 94:5594–5598.

Folk, J.E. 1983. Mechanism and basis for specificity of transglutaminase-cata- lyzed epsilon-(gamma-glutamyl) lysine bond formation. Adv. Enzymol. Relat. Areas Mol. Biol. 54:51–56.

Green, H. 1993. Human genetic diseases due to codon reiteration: relationship to an evolutionary mechanism. Cell. 74:955–956.

Greenberg, C.S., P.J. Birckbichler, and R.H. Rice. 1991. Transglutaminases: multifunctional cross-linking enzymes that stabilize tissues. FASEB J. 5:3303–3307.
ment. Neurobiol. Dis. 6:364–375.

Perutz, M. 1994. Polar zippers: their role in human disease. Prot. Sci. 3:1629–1637.

Perutz, M.F., T. Johnson, M. Suzuki, and J.T. Finch. 1994. Glutamine repeats as polar zippers: their possible role in inherited neurodegenerative diseases. Proc. Natl. Acad. Sci. USA. 91:5355–5358.

Preis, P.N., H. Saya, L. Nadasdi, G. Hochhaus, V. Levin, and W. Sadee. 1988. Neuronal cell differentiation of human neuroblastoma cells by retinoic acid plus herbimycin A. Cancer Res. 48:6530–6534.

Saudou, F., S. Finkbeiner, D. Doxys, and M.E. Greenberg. 1998. Huntingtin acts in the nucleus to induce apoptosis but death does not correlate with the formation of intranuclear inclusions. Cell. 95:55–66.

Sawa, A., G. Wiegand, J. Cooper, R. Margolis, A. Sharp, J. Lawler, Jr., J. Greenamyer, S. Snyder, and C. Ross. 1999. Increased apoptosis of Huntington disease lymphoblasts associated with repeat length-dependent mitochondrial depolarization. Nat. Med. 5:1194–1198.

Scherzinger, E., R. Lurz, M. Turmaine, L. Mangiarini, B. Hollenbach, R. Hasenbank, G.P. Bates, S.W. Davies, H. Lehrach, and E.E. Wanker. 1997. Huntington-encoded polyglutamine expansions form amyloid-like protein aggregates in vitro and in vivo. Cell. 90:549–558.

Schilling, G., M.W. Becher, A.H. Sharp, H.A. Jinnah, K. Duan, J.A. Kotzuk, H.H. Slunt, T. Ratovitski, J.K. Cooper, N.A. Jenkins, et al. 1999. Intranuclear inclusions and neuritic aggregates in transgenic mice expressing a mutant N-terminal fragment of huntingtin. Hum. Mol. Genet. 8:397–407.

Stott, K., J.M. Blackburn, P.J. Butler, and M. Perutz. 1995. Incorporation of glutamine repeats makes protein oligomerize: implications for neurodegenerative diseases. Proc. Natl. Acad. Sci. USA. 92:6509–6513.

Tucholski, J., M. Lesort, and G.V.W. Johnson. 2001. Tissue transglutaminase is essential for neurite outgrowth in human neuroblastoma SH-SY5Y cells. Neuroscience. 102:481–491.

Wheeler, V.C., J.K. White, C.A. Gutekunst, V. Vrbanac, M. Weaver, X.J. Li, S.H. Li, H. Yi, J.P. Vonsattel, J.F. Gusella, et al. 2000. Long glutamine tracts cause nuclear localization of a novel form of huntingtin in medium spiny striatal neurons in HdhQ92 and HdhQ111 knock-in mice. Hum. Mol. Genet. 9:503–513.

Wyttenbach, A., J. Carmichael, J. Swartz, R.A. Furlong, Y. Narain, J. Rankin, and D.C. Rubinsztein. 2000. Effects of heat shock, heat shock protein 40 (HDJ-2), and proteasome inhibition on protein aggregation in cellular models of Huntington’s disease. Proc. Natl. Acad. Sci. USA. 97:2898–2903.

Yamamoto, A., J.J. Lucas, and R. Hen. 2000. Reversal of neuropathology and motor dysfunction in a conditional model of Huntington’s disease. Cell. 101:57–66.

Zhang, J., M. Lesort, R.P. Guttmann, and G.V. Johnson. 1998. Modulation of the in situ activity of tissue transglutaminase by calcium and GTP. J. Biol. Chem. 273:2288–2295.