Method

1. Heat two 15-ml conical polypropylene tubes containing 5 ml of distilled water to 50°. To each tube, add 5 mg of gelatin and swirl to dissolve.
2. To one tube, add 125 μl of the aggregate suspension prepared earlier. Vortex the aggregate stock suspension well before diluting.
3. Place a 50-μl drop of the aggregate gelatin suspension onto a gelatin-subbed slide. About 3 separate drops will fit on each slide. Agitate the tube containing the suspension often to avoid settling.
4. To the second tube containing gelatin, add 125 μl of aggregation buffer (protocol 3). Using this solution, prepare polymer-treated slides that contain no Aβ aggregates. These slides will serve as negative controls and for determining background binding. Dry the slides in a 50° incubator until all of the liquid has evaporated (1–2 hr). Store the slides in a dry environment until use.

Deposition Assay Protocol

Materials

Synthetic amyloid slides:	Prepared as described earlier or obtained from commercial sources (QCB), Hopkinton, MA)
Deposition buffer:	50 m*M* Tris-HCl, pH 7.5, at room temperature containing 0.1% (w/v) BSA
Wash buffer:	50 m*M* Tris-HCl, pH 7.5, at room temperature
Radiolabeled Aβ:	[^{125}I]Aβ (prepared as described in text or obtained from commercial sources)

Method. Steps 1 through 5 as in protocol 1.

Note: For all these protocols, compounds to be tested for effects on Aβ deposition rate (candidate accelerants or inhibitors) may be added to preincubations, incubations, or both.[10] The assays described here are quite tolerant of organic solvents, e.g., all perform well in 10% (v/v) dimethyl sulfoxide.

Acknowledgments

We thank D. Selkoe for the gift of anti-Aβ antiserum and H. Vinters for the gift of human brain tissue. Work in the authors' laboratories has been supported by the National Institutes of Health, the American Health Assistance Foundation, and the Veterans' Administration.

[24] Membrane Filter Assay for Detection of Amyloid-like Polyglutamine-Containing Protein Aggregates

By ERICH E. WANKER, EBERHARD SCHERZINGER, VOLKER HEISER, ANNIE SITTLER, HOLGER EICKHOFF, and HANS LEHRACH

Introduction

The accumulation of polyglutamine-containing protein aggregates in neuronal intranuclear inclusions (NIIs) has been demonstrated for several progressive neurodegenerative diseases such as Huntington's disease (HD),[1,2] dentatorubral pallidoluysian atrophy (DRPLA),[2,3] and spinocerebellar ataxia (SCA) types 1,[4,5] 3,[6] and 7.[7] Furthermore, it has been shown *in vitro* that the proteolytic cleavage of fusion proteins of glutathione *S*-transferase (GST) and the polyglutamine-containing huntingtin peptide coded for by the first exon of the HD gene[8] leads to the formation of insoluble high molecular weight protein aggregates with a fibrillar or ribbon-like morphology[9] reminiscent of β-amyloid fibrils in Alzheimer's disease and scrapie prion rods.[10,11]

Insoluble, ordered protein aggregates (amyloids) are commonly ana-

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lyzed by electron microscopy (EM),[9,12] sedimentation,[13] fluorescence staining,[14] turbidity,[15] or quasi-elastic light scattering (QLS).[16,17] All these existing methods have complementary strengths and weaknesses. EM, for instance, is useful for the identification of amyloid fibrils and determining their morphology, but this technique is not suitable for the quantification of amyloids because of the clumping of fibrils.[9] Sedimentation has been used to separate insoluble amyloid fibrils from soluble monomeric protein. However, this method does not distinguish ordered amyloid fibrils from insoluble amorphous aggregates.[13,18] The thioflavin fluorescence assay has been widely used to examine the formation of Aβ amyloids *in vitro*,[14,19] but a general application of this assay for the detection of other amyloid-like aggregates such as huntingtin fibrils has not been demonstrated. This is because not all ordered protein aggregates bind thioflavin. Both turbidity[15] and QLS[16,17] measurements have been used successfully for the quantification of amyloid fibrils formed *in vitro*. However, they are not specific for amyloids. Also, relatively large amounts of recombinant proteins or peptides are needed for the quantification of fibrillar structures with these methods.

For the detection and quantification of small amounts of polyglutamine-containing protein aggregates we have developed a rapid and sensitive filter retardation assay, which should be suitable for high-throughput screenings of drugs that prevent aggregate formation. This assay is based on the finding that the polyglutamine-containing protein aggregates are insoluble in sodium dodecyl sulfate (SDS) and are retained on a cellulose acetate filter, whereas the monomeric forms of the HD exon 1 protein do not bind to this filter membrane. The captured aggregates are then detected by simple immunoblot analysis using specific antibodies. This article describes in detail the use of the filter retardation assay for the identification and quantification of huntingtin protein aggregates formed *in vitro* and *in vivo* in a transient expression system using COS cells.

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Construction of Plasmids

Standard protocols for DNA manipulations are followed.[20] *IT-15* cDNA sequences[8] encoding the N-terminal portion of huntingtin, including the CAG repeats, are amplified by polymerase chain reactions (PCR) using the oligonucleotides ES25 (5′-TGGGATCCGCATGGCGACCCTGG AAAAGCTGATGAAGG-3′) and ES27 (3′-CTCCTCGAGCGGCGG TGGCGGCTGTTGCTGCTGCTGCTG-5′) as primers and the plasmids pCAG20 and pCAG51 as template.[9] Conditions for PCR are as described.[21] The resulting cDNA fragments are gel purified, digested with *Bam*HI and *Xho*I, and inserted into the *Bam*HI–*Xho*I site of the expression vector pGEX-5X-1 (Pharmacia, Piscataway, NJ), yielding pCAG20ΔP and pCAG51ΔP, respectively. The plasmids pCAG20ΔP-Bio and pCAG51ΔP-Bio are generated by subcloning the PCR fragments obtained from the plasmids pCAG20 and pCAG51 into pGEX-5X-1-Bio. pGEX-5X-1-Bio is created by ligation of the oligonucleotides BIO1 (5′-CGCTCGAGGG TATCTTCGAGGCCC AGAAGATCGAGTGGCGATCACCATGAG C-3′) and BIO2 (5′-GGCCGCTCATGGTGATCGCCACTCGATCTTCT GGGCCTCGAAGATACCCTCGAGCG-3′), after annealing and digestion with *Xho*I, into the *Xho*I–*Not*I site of pGEX-5X-1. Plasmids with the *IT-15* cDNA inserts are sequenced to confirm that no errors have been introduced by PCR. The construction of plasmids pTL1-CAG20, pTL1-CAG51, and pTL1-CAG93 for the expression of huntingtin exon 1 proteins containing 20, 51, and 93 glutamines in mammalian cells has been described.[22]

Structure of GST-HD Fusion Proteins

The amino acid sequence of the GST-HD fusion proteins encoded by the *Escherichia coli* expression plasmids pCAG20ΔP, pCAG51ΔP, pCAG20ΔP-Bio, and pCAG51ΔP-Bio is shown in Fig. 1. The plasmids pCAG20ΔP and pCAG51ΔP encode fusion proteins of GST and the N-terminal portion of huntingtin containing 20 (GST-HD20ΔP) and 51 (-HD51ΔP) polyglutamines, respectively. In these proteins the proline-rich region located immediately downstream of the glutamine repeat is deleted.[9]

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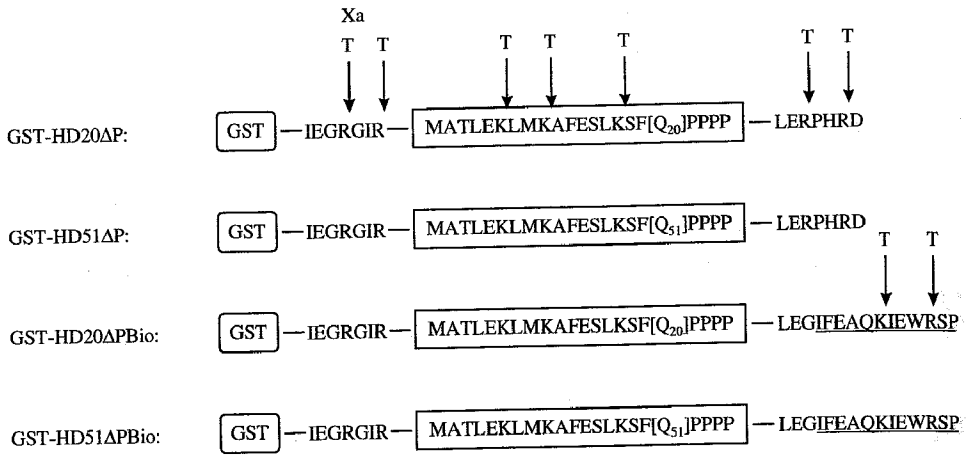

FIG. 1. Structure of GST-HD fusion proteins. The amino acids sequence corresponding to the N-terminal portion of huntingtin is boxed and the amino acids corresponding to the biotinylation site are underlined. Arrows labeled (Xa) and (T) indicate cleavage sites for factor Xa and trypsin, respectively.

The fusion proteins GST-HD20ΔPBio and -HD51ΔPBio are identical to GST-HD20ΔP and -HD51ΔP, except for the presence of a biotinylation site[23] at their C termini.

Strains and Media

Escherichia coli DH10B (BRL) is used for plasmid construction, and *E. coli* SCS1 (Stratagene) is used for the expression of GST-HD fusion proteins. Transformation of *E. coli* with plasmids and ligation mixtures is performed by electroporation using a Bio-Rad Gene Pulser (Richmond, CA). Transformed cells are spread on LB plates supplemented with appropriate antibiotics.[20] For expression of GST fusion proteins, cells are grown in liquid TY medium (5 g NaCl, 5 g yeast extract, and 10 g tryptone per liter) buffered with 20 m*M* MOPS/KOH (pH 7.9) and supplemented with glucose (0.2%), thiamine (20 μg/ml), and ampicillin (100 μg/ml). COS-1 cells are grown in Dulbecco's modified Eagle's medium (GIBCO-BRL) supplemented with 5% (w/v) fetal calf serum (FCS) containing penicillin (5 U/ml) and strepomycin (5 μg/ml); transfection is performed as described.[24]

Purification of GST Fusion Proteins

The procedure for the purification of GST fusion proteins is an adaption of the protocol of Smith and Johnson.[25] Unless indicated otherwise, all steps are performed at 0–4°.

1. Inoculate 100 ml TY medium with a single colony containing the expression plasmid of interest and incubate the culture at 37° overnight with shaking.
2. Inoculate 1.5 liter TY medium with the overnight culture and grow at 37° until an OD_{600} of 0.6 is reached.
3. Add isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 m*M* and continue to grow the culture at 37° for 3.5 hr with vigorous shaking.
4. Chill the culture on ice, and harvest the cells by centrifugation at 4000*g* for 20 min.
5. Wash cells with buffer A [50 m*M* sodium phosphate (pH 8), 150 m*M* NaCl, and 1 m*M* EDTA] and, if necessary, store the cell pellet at −70°.
6. Resuspend cells in 25 ml buffer A, add phenylmethylsulfonyl fluoride (PMSF) and lysozyme (Boehringer Mannheim) to 1 m*M* and 0.5 mg/ml, respectively, and incubate on ice for 45 min.
7. Lyse cells by sonication (2 × 45 sec, 1 min cooling, 200–300 W), and add Triton X-100 to a final concentration of 0.1% (v/v).
8. Centrifuge the lysate at 30,000*g* for 30 min and collect the supernatant.
9. Add 5 ml of a 1:1 slurry of GST-agarose (Sigma, St. Louis, MO), equilibrated previously in buffer A, and stirr the mixture for 30 min.
10. Pour the slurry into a 1.6-cm-diameter column, wash once with 40 ml buffer A containing 1 m*M* (PMSF) and 0.1% Triton X-100, and wash twice with 40 ml buffer A containing 1 m*M* PMSF.
11. Elute the protein with 5 × 2 ml buffer A containing 15 m*M* reduced glutathione (Sigma). Analyze aliquots of the fractions by SDS–PAGE and combine the fractions containing the purified GST fusion protein.
12. Dialyze the pooled fractions overnight against buffer B [20 m*M* Tris–HCl (pH 8), 150 m*M* NaCl, 0.1 m*M* EDTA, and 5% (v/v) glycerol], aliquot, freeze in liquid nitrogen, and store at −70°.

Typical yields are 10–20 mg for GST-HD20ΔP and -HD51ΔP and 5–10 mg for GST-HD20ΔPBio and-HD51ΔPBio per liter of bacterial culture. The protein concentration is determined using the Coomassie protein assay reagent from Pierce with bovine serum albumin (BSA) as a standard.

Proteolytic Cleavage of GST Fusion Proteins

The GST–huntingtin fusion proteins (2 μg) are digested with bovine factor Xa (New England Biolabs) or with modified trypsin (Boehringer Mannheim, sequencing grade) at an enzyme/substrate ratio of 1:10 (w/w) and 1:20 (w/w), respectively. The reaction is carried out in 20 μl of 20 m*M* Tris–HCl (pH 8), 150 m*M* NaCl, and 2 m*M* $CaCl_2$. Incubations with factor Xa are performed at 25° for 16 hr. Tryptic digestions are at 37° for 3 to 16 hr. Digestions are terminated by the addition of 20 μl 4% (w/v) SDS and 100 m*M* dithiothreitol (DTT), followed by heating at 98° for 5 min.

Isolation of Amyloid-like Protein Aggregates from Transfected COS-1 Cells

COS-1 cells transfected with the mammalian expression plasmids are harvested 48 hr after transfection. The cells are washed in ice-cold phosphate-buffered saline (PBS), scraped, and pelleted by centrifugation (2000*g*, 10 min, 4°). Cells are lysed on ice for 30 min in 500 μl lysis buffer [50 m*M* Tris–HCl (pH 8.8), 100 m*M* NaCl, 5 m*M* $MgCl_2$, 0.5% (w/v) Nonidet P-40 (NP-40), 1 m*M* EDTA] containing the protease inhibitors PMSF (2 m*M*), leupeptin (10 μl/ml), pepstatin (10 μg/ml), aprotinin (1 μg/ml), and antipain (50 μg/ml). Insoluble material is removed by centrifugation for 5 min at 14,000 rpm in a microfuge at 4°. Pellets containing the insoluble material are resuspended in 100 μl DNase buffer [20 m*M* Tris–HCl (pH 8.0), 15 m*M* $MgCl_2$], and DNase I (Boehringer Mannheim) is added to a final concentration of 0.5 mg/ml followed by incubation at 37° for 1 hr. After DNase treatment the protein concentration is determined by the dot metric assay (Geno Technology) using BSA as a standard. Incubations are terminated by adjusting the mixtures to 20 m*M* EDTA, 2% (w/v) SDS, and 50 m*M* DTT, followed by heating at 98° for 5 min.

Dot-Blot Filter Retardation Assay

The filter assay used to detect polyglutamine-containing huntingtin protein aggregates has been described.[9] Denatured and reduced protein samples are prepared as described above, and aliquots corresponding to 50–250 ng fusion protein (GST-HD20ΔP and GST-HD51ΔP) or 5–30 μg extract protein (pellet fraction) of COS-1 cells are diluted into 200 μl 2% SDS and filtered on a BRL dot-blot filtration unit through a cellulose acetate

been preequilibrated with 2% SDS. Filters are washed twice with 200 μl 0.1% SDS and are then blocked in TBS (100 m*M* Tris–HCl, pH 7.4, 150 m*M* NaCl) containing 3% nonfat dried milk, followed by incubation with the anti-HD1 antibody (1:1000).[9] The filters are washed several times in TBS and are then incubated with a secondary anti-rabbit antibody conjugated to horseradish peroxidase (Sigma, 1:5000) followed by ECL (enhanced chemiluminescence, Amersham) detection. The developed blots are exposed for various times to Kodak (Rochester, NY) X-OMAT film or to a Lumi-Imager (Boehringer Mannheim) to enable quantification of the immunoblots.

For detection and quantification of polyglutamine-containing aggregates generated from the protease-treated fusion proteins GST-HD20ΔPBio and -HD51ΔPBio, the biotin/streptavidin–AP detection system is used. Following filtration, the cellulose acetate membranes are incubated with 1% (w/v) BSA in TBS for 1 hr at room temperature with gentle agitation on a reciprocal shaker. Membranes are then incubated for 30 min with streptavidin–alkaline phosphatase (Promega, Madison, WI) at a 1:1000 dilution in TBS containing 1% BSA, washed three times in TBS containing 0.1% (v/v) Tween 20 and three times in TBS, and finally incubated for 3 min with either the fluorescent alkaline phosphatase substrate AttoPhos or the chloro-substituted 1,2-dioxetane chemiluminescence substrate CDP-Star (Boehringer Mannheim) in 100 m*M* Tris–HCl, pH 9.0, 100 m*M* NaCl, and 1 m*M* $MgCl_2$. Fluorescent and chemiluminescent signals are imaged and quantified with the Boehringer Lumi-Imager F1 system and LumiAnalyst software (Boehringer Mannheim).

Results

As shown previously,[9] removal of the GST tag from the HD exon 1 protein containing 51 glutamines (GST-HD51) by site-specific proteolytic cleavage results in the formation of high molecular weight protein aggregates, seen as characteristic fibrils or filaments on electron microscopic examination. Such ordered fibrillar structures were not detected after proteolysis of fusion proteins containing only 20 (GST-HD20) or 30 (GST-HD30) glutamines, although light-scattering measurements[17] revealed that some form of aggregation also occurred with these normal repeat-length proteins. In the present study, truncated GST-HD exon 1 fusion proteins with or without a C-terminal biotinylation tag[23] were used. These fusion proteins contain either 20 or 51 glutamines but lack most of the proline-rich

sin cleavage sites within the GST-HD fusion proteins are shown in Fig. 1. The proteins GST-HD20ΔP and -HD51ΔP were expressed in *E. coli* and affinity-purified under native conditions. They were then digested overnight with trypsin or factor Xa protease to promote the formation of polyglutamine-containing huntingtin aggregates. Figure 2A shows an immunoblot of a cellulose acetate membrane to which the native GST-HD20ΔP and -HD51ΔP proteins and their factor Xa and trypsin cleavage products have been applied. As expected from our previous studies using fusions of GST and the full-length HD exon 1 protein,[9] only the cleavage products of GST-HD51ΔP were retained by the filter and were detected by the huntingtin-specific antibody HD1, indicating the formation of high molecular weight protein HD51ΔP aggregates from this fusion protein. Scanning electron microscopy of the material retained on the surface of the membrane revealed bunches of long fibrils or filaments (Fig. 2B), which were not detected after filtration of the uncleaved GST-HD51ΔP preparation or the protease-treated GST-HD20ΔP preparation. These results indicate that an elongated polyglutamine sequence but not the proline-rich region in the HD exon 1 protein is necessary for the formation of high molecular weight protein aggregates *in vitro*.

To examine whether polyglutamine-containing aggregates are also formed *in vivo,* HD exon 1 proteins with 20, 51, or 93 glutamines (without a GST tag) were expressed in COS-1 cells. Whole cell lysates were prepared, and after centrifugation, the insoluble material was collected and treated with DNase I to achieve maximal resuspension. The resulting protein mixture was then boiled in SDS and analyzed using the dot-blot filter retardation assay. Figure 2C shows that insoluble protein aggregates are being formed in transfected COS cells expressing the HD exon 1 protein with 51 and 93 glutamines but not in COS cells expressing the normal exon 1 allele with 20 glutamines or in nontransfected control cells. Thus, as observed *in vitro* with purified GST fusion proteins, the formation of high molecular weight aggregates *in vivo* occurs in a repeat length-dependent way and requires a polyglutamine repeat in the pathological range. In addition, like the *in vitro* aggregates, HD exon 1 aggregates formed *in vivo* are resistant to boiling in 2% (w/v) SDS as well as to 8 *M* urea.

To monitor the *in vitro* formation of polyglutamine-containing aggregates without the need for a specific antibody, a modified filter retardation assay was developed. In this assay, streptavidin-conjugated alkaline phosphatase (AP) is used to detect the insoluble protein aggregates retained on the cellulose acetate filter membrane. Streptavidin binds specifically to the biotinylation tag[23] that has been added C-terminal to the polyglutamine tract in the fusion proteins GST-HD20ΔPBio and -HD51ΔPBio (Fig. 1).

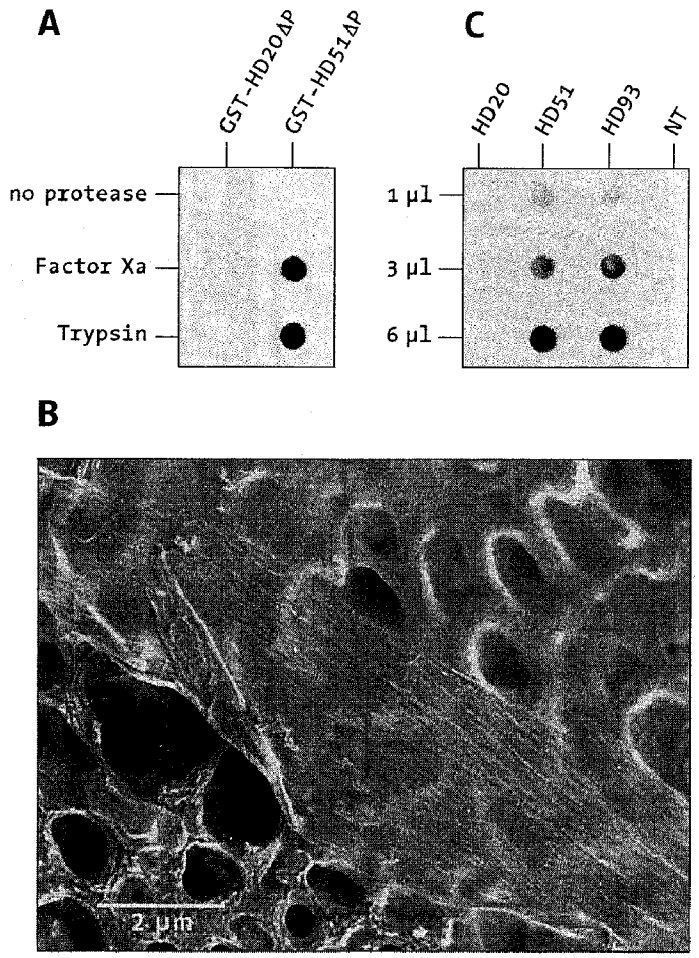

FIG. 2. Detection of polyglutamine-containing protein aggregates formed *in vitro* and in transfected COS-1 cells using the dot-blot filter retardation assay. (A) Purified GST-HD20ΔP and -HD51ΔP fusion proteins (250 ng) and their factor Xa and trypsin cleavage products were applied to the filter as indicated. The aggregated proteins retained by the cellulose acetate membrane were detected by incubation with the anti-HD1 antibody. (B) Scanning electron micrograph of aggregated GST-HD51ΔP trypsin cleavage products retained on the surface of the cellulose acetate membrane (Heinrich Lündsdorf, GBF Braunschweig, Germany). (C) Dot-blot filter retardation assay performed on the insoluble fraction isolated from transfected and nontransfected COS-1 cells. COS-1 cells were transfected transiently with the plasmids pTL1-CAG20, -CAG51, and -CAG93 encoding huntingtin exon 1 proteins with 20 (HD20), 51 (HD51), and 93 (HD93) glutamines, respectively. The pellet fractions obtained after centrifugation of whole cell lysates were subjected to DNase I digestion, boiled in 2% SDS, and portions of 1, 3, and 6 µl were filtered through a cellulose acetate membrane. The aggregated huntingtin protein retained on the membrane was detected with the anti-HD1 antibody. NT, nontransfected cells.

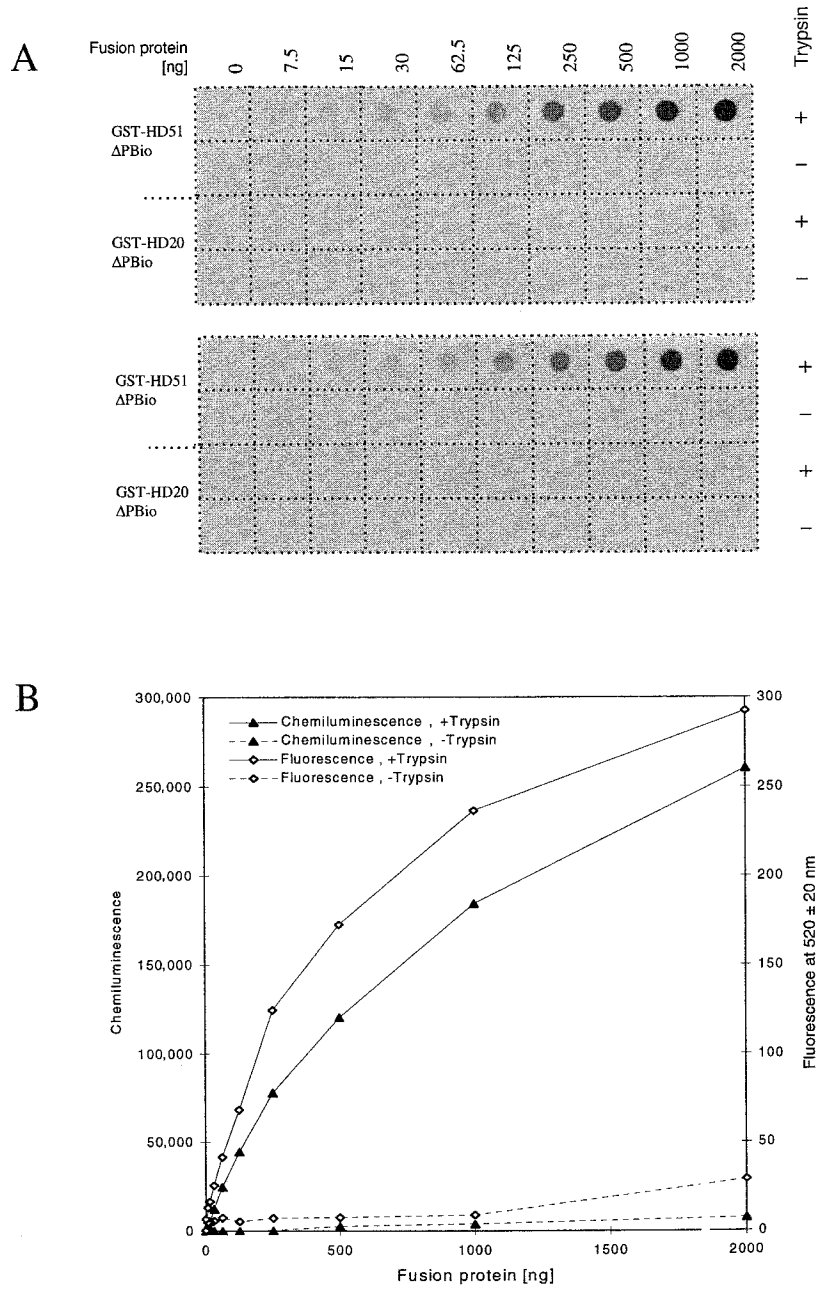

Figure 3A shows that the modified aggregation assay gives results comparable to those obtained with nonbiotinylated fusion proteins in that insoluble aggregates are produced from the trypsin-treated GST-HD51ΔPBio protein but not from the uncleaved GST-HD51ΔPBio protein or the corresponding 20 repeat samples. Using either fluorescent (AttoPhos) or chemiluminescent (CDP-Star) substrates for alkaline phosphatase, it is possible to capture and quantify the filter assay results with the Boehringer Lumi-Imager F1 system. With both AP substrates, aggregates formed from as little as 5–10 ng of input GST-HD51ΔPBio protein were readily detected on the cellulose acetate membrane, and signal intensities increased linearly up to 250 ng of fusion protein applied to the filter (Fig. 3B).

Conclusions

Our results demonstrate that the cellulose acetate filter retardation assay can be a useful tool for the identification, structural characterization, and quantification of SDS-insoluble polyglutamine-containing protein aggregates formed *in vitro* and *in vivo*. In addition to the histochemical identification of amyloids, this assay may be useful in detecting insoluble protein aggregates in all types of human and animal amyloidoses, including the polyglutamine diseases, and also in screening compound libraries for potential aggregation inhibitors. Currently, attempts to develop a microtiter plate-based high-throughput filter retardation assay to identify chemical compounds that slow down the rate of formation of polyglutamine-containing fibrils *in vitro* are in progress. The amyloid-binding agents arising from this screen then will be tested in a HD cell culture model system and in the HD animal model[21,26] for their therapeutic potential.

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FIG. 3. Detection and quantification of aggregates formed *in vitro* from biotinylated GST-HD exon 1 fusion proteins. Various amounts of the fusion proteins GST-HD51ΔPBio and -HD20ΔPBio were filtered through a cellulose acetate membrane after a 3-hr incubation at 37° in the presence or absence of trypsin as indicated. (A) Images of the retained protein aggregates, detected with streptavidin–AP conjugate using either a fluorescent (top) or a chemiluminescent AP substrate (bottom). (B) Quantification of signal intensities obtained for the GST-HD51ΔPBio dots seen in A. Fluorescence and chemiluminescence values are arbitrary units generated by the Lumi-Imager F1 and LumiAnalyst software (Boehringer Mannheim).