Hsp20, a novel α -crystallin, prevents A β fibril formation and toxicity

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(RECEIVED August 31, 2004; FINAL REVISION November 9, 2004; ACCEPTED November 10, 2004)

Abstract

β -Amyloid (A β) is a major protein component of senile plaques in Alzheimer's disease, and is neurotoxic when aggregated. The size of aggregated A β responsible for the observed neurotoxicity and the mechanism of aggregation are still under investigation; however, prevention of A β aggregation still holds promise as a means to reduce A β neurotoxicity. In research presented here, we show that Hsp20, a novel α -crystallin isolated from the bovine erythrocyte parasite *Babesia bovis*, was able to prevent aggregation of denatured alcohol dehydrogenase when the two proteins are present at near equimolar levels. We then examined the ability of Hsp20 produced as two different fusion proteins to prevent A β amyloid formation as indicated by Congo Red binding; we found that not only was Hsp20 able to dramatically reduce Congo Red binding, but it was able to do so at molar ratios of Hsp20 to A β of 1 to 1000. Electron microscopy confirmed that Hsp20 does prevent A β fibril formation. Hsp20 was also able to significantly reduce A β toxicity to both SH-SY5Y and PC12 neuronal cells at similar molar ratios. At high concentrations of Hsp20, the protein no longer displays its aggregation inhibition and toxicity attenuation properties. Size exclusion chromatography indicated that Hsp20 was active at low concentrations in which dimer was present. Loss of activity at high concentrations was associated with the presence of higher oligomers of Hsp20. This work could contribute to the development of a novel aggregation inhibitor for prevention of A β toxicity.

Keywords: Alzheimer's disease; protein folding; chaperone; amyloid; aggregation

Alzheimer's disease (AD), the leading cause of dementia in the aging population, is accompanied by the accumulation of β -amyloid peptide (A β) as amyloid fibrils in senile plaques in the cerebral cortex. It is hypothesized by many that A β contributes to the neurodegeneration associated with AD via the toxicity of the peptide in aggregated form.

Investigation of the relationship between aggregated A β peptide size, structure, and toxicity is ongoing. In an aggregated state containing fibrils, protofibrils, and low molecular weight intermediates/oligomers, A β peptide has proven

to be toxic to cultured neuronal cells (Koh et al. 1990; Pike et al. 1993; Lambert et al. 1998; Kaye et al. 2003). A β structures reported to be toxic include a nonfibrillar species of ~17–42 kDa (Lambert et al. 1998; Chromy et al. 2003), protofibrils species with hydrodynamic radii on the order of 9–367 nm (Ward et al. 2000; Wang et al. 2002), and fibril species, with some investigators suggesting that the smaller oligomeric A β species are more toxic than fibril and protofibril forms (Dahlgren et al. 2002; Chromy et al. 2003; Kaye et al. 2003). Many believe that one strategy for preventing neurodegeneration associated with AD is the prevention of aggregation of A β into its toxic oligomeric or fibril forms.

A number of investigators have developed inhibitors of A β aggregation with the aim of preventing A β toxicity (Ghanta et al. 1996; Lansbury 1997; Pallitto et al. 1999;

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Article and publication date are at <http://www.proteinscience.org/cgi/doi/10.1110/ps.041020705>.

Lowe et al. 2001). Some of these inhibitors have been small peptides with sequences that mimic the sequence of A β believed to be essential for aggregation and fibril formation (Pallitto et al. 1999). Peptide inhibitors of this class have been able to prevent A β toxicity by altering the aggregated structure when added at inhibitor to A β molar ratios of near 1:1 (Tjernberg et al. 1996; Pallitto et al. 1999). The use of molecular chaperones including α -crystallins and other small heat shock proteins has also been explored as a means of preventing A β aggregation and toxicity.

The superfamily of small heat shock proteins, sHSPs, are a diverse group of proteins involved in prevention of protein misfolding and the onset of programmed cell death (van den Ijssel et al. 1999; Bruey et al. 2000). They typically exist as large multimeric complexes containing four to 40 subunits, with molecular masses of 40 kDa or less per subunit. The sHSPs bind to their substrates with high affinity and prevent protein misfolding in an ATP-independent reaction (Jakob et al. 1993). Generally, the α -crystallins are considered to be sHSPs (Horwitz 1992; MacRae 2000). β -Pleated sheet dominates their secondary structure (Horwitz 2000; van Montfort et al. 2001). A conserved 90-amino-acid sequence, located near the C-terminus, termed the α -crystallin domain, is the hallmark of all α -crystallins.

Small heat shock proteins including α -crystallins have been tested for A β aggregation and toxicity prevention activity, mixed results have been reported. Human sHsp27 inhibited A β 1–42 fibril formation (Kudva et al. 1997). When α B crystallin was examined, it was actually found to increase toxicity and β pleated sheet content of A β 1–40 although it prevented fibril formation of A β in vitro at α -crystallin to A β molar ratios of 1:1 or 1:5 (Stege et al. 1999).

In work described here, we use a novel small heat shock protein, Hsp20, isolated from the erythrocyte parasite *Ba-*

besia bovis to prevent aggregation of A β and A β toxicity. Using two different Hsp20 constructs, a maltose binding protein fusion (Hsp20-MBP) and a polyhistidine fusion (his-Hsp20), we show that Hsp20 has α -crystallin-like properties, and that it can prevent aggregation of denatured alcohol dehydrogenase. Hsp20 is able to prevent amyloid formation of A β as indicated by Congo Red binding at molar ratios of Hsp20 to A β of 1:1000. Hsp20 attenuates the toxicity of A β in SH-SY5Y and PC12 neuronal cells at analogous molar ratios. Hsp20 appears to be able to prevent A β aggregation via a novel mechanism and at much lower concentrations than what has been necessary to prevent aggregation with other inhibitors. Hsp20 may be a useful molecular model for the design of the next generation of A β aggregation inhibitors to be used in prevention of A β toxicity.

Results

Characterization of Hsp20

Hsp20 is a 177-amino-acid, 20.1-kDa protein isolated from *Babesia bovis*, a protozoan bovine erythrocyte parasite (Brown et al. 2001). Initial characterization by BLAST search of the NCBI database indicated significant sequence similarity with the α -crystallin family of small heat shock proteins. Hsp20 contains a region near its C-terminus that corresponds to the ~95 amino acid α -crystallin domain common to members of this protein family. These proteins are thought to be involved in the cellular response to stress. Previous work (A. Rice-Ficht, unpubl.) has revealed that Hsp20 expression is up-regulated in times of thermal, nutritional, and oxidative stress to the organism. Based on the apparent involvement of Hsp20 in the stress response coupled with its homology to the α -crystallins, α -crystallin activity assays were applied to Hsp20. A turbidity assay (Fig. 1) was used to quantitate the ability of Hsp20-MBP to solubilize target proteins at elevated temperatures. A solution of alcohol dehydrogenase when incubated at 58°C for 60 min exhibited a dramatic increase in light scattering due to aggregation of the enzyme. When Hsp20-MBP was included in the solution at time zero in a molar ratio of 2:1 Hsp20:ADH, a 2.3-fold reduction in light scattering was observed at 60 min. To determine the most effective molar ratio for the reduction of light scattering, a series of experiments was conducted in which different mole ratios of Hsp20 were added to ADH (Fig. 2). At time infinity (determined by curve fitting software), the greatest reduction in light scattering was seen at a molar ratio of 2:1 (Hsp20 to ADH). Since Hsp20-MBP was prepared as a fusion protein with maltose binding protein, we examined the ability of maltose binding protein (MBP) alone to alter aggregation and turbidity of ADH in the same range of concentrations. MBP had no effect on ADH aggregation, suggesting the aggregation-prevention activity was due to the presence of

Figure 1. Light scattering of ADH, as determined by absorbance at 360 nm, at elevated temperature in the presence and absence of Hsp20-MBP. ADH at a concentration of 1.625 μ M is incubated in phosphate buffer alone (open circles) and in the presence of 5.75 μ M Hsp20 (closed triangles) at 58°C for 1 h.

Figure 2. Percent reduction of light scattering at time infinity of ADH when mixed with Hsp20-MBP relative to light scattering of ADH alone. The negative of light scattering seen at 2:1 molar ratio (Hsp20:ADH) indicates that light scattering of the Hsp20:ADH mixture was 80% less than light scattering of ADH alone. The near zero value of change in light scattering at 1:1000 Hsp20:ADH mole ratio indicates that light scattering of the mixture was unchanged from light scattering of ADH seen in Figure 1. All experiments were performed at 58°C. The concentration of ADH was 1.625 μ M in each experiment. Light scattering at time infinity was estimated from absorbance versus time curves for each mixture.

Hsp20. Having established the α -crystallin activity of Hsp20-MBP based on standard assays, we then proceeded to test the ability of Hsp20-MBP to affect A β (1–40) fibril formation.

Effect of Hsp20-MBP on A β fibril formation prevention

Given that Hsp20-MBP was able to prevent aggregation of denatured ADH, we then examined its ability to prevent aggregation and amyloid fibril formation of A β (1–40). We used Congo Red binding as a simple indicator of amyloid formation. In these experiments, 20 μ M, 50 μ M, and 100

Figure 3. Representative absorbance spectra of Congo Red with A β and A β -Hsp20 mixtures. One-hundred-millimolar A β (1–40) was used in all cases. Hsp20-MBP was added at the beginning of the 24-h aggregation at room temperature with mixing. (Bold line, Congo Red alone; fine line, A β alone; light gray line, 1:100 Hsp20:A β ; dark gray line, 1:1000 Hsp20:A β .)

μ M concentrations of A β (1–40) were used to create fibrils. Hsp20-MBP at concentrations from 1 nM to 5 μ M were added to A β (1–40) solutions prior to aggregation. This corresponds to molar ratios of Hsp20-MBP to A β of 1:20,000 to 1:20 or 0.00005 to 0.05. After 24 h of incubation, sufficient time for typical fibril formation, Congo Red was added to samples to assess fibril formation. Representative Congo Red absorbance spectra are seen in Figure 3. Hsp20-MBP alone did not alter Congo Red absorbance. Pure A β (1–40) solutions caused a characteristic shift in absorbance to longer wavelengths and an increase in intensity. Hsp20-MBP, when added to A β prior to aggregation, attenuated the shift in absorption and increase in intensity associated with A β fibril formation. MBP alone, when added to A β at similar concentrations had no effect on fibril formation as assessed by Congo Red binding.

We show in Figure 4A the effect of Hsp20-MBP on reduction of A β fibril formation at different concentrations of A β and Hsp20-MBP. At Hsp20-MBP concentrations below 0.1 μ M, A β fibril formation decreased as a function of Hsp20-MBP concentration. However, Hsp20-MBP became less effective at preventing A β fibril formation in the higher concentration range. The concentration of Hsp20-MBP needed for optimal prevention of fibril formation appeared

Figure 4. Relative fibril formation as estimated from Congo Red binding. Fibril formation is reported relative to fibril formation seen with pure A β solutions. Mean plus or minus standard deviation of at least three independent experiments are reported. (A) Relative fibril formation as a function Hsp20-MBP concentration. (B) Relative fibril formation as a function of the molar ratio of Hsp20-MBP to A β . Open circles, 20 μ M A β ; squares, 50 μ M A β ; triangles, 100 μ M A β .

to be a function of A β concentration, with the lowest concentration of A β exhibiting the optimum in prevention of fibril formation at the lowest Hsp20-MBP concentration. In Figure 4B, we replotted the fibril formation data as a function of molar ratio of Hsp20-MBP to A β at the three different A β concentrations. It would appear from Figure 4B that the three curves are now superimposed. The optimum in fibril formation for the three concentrations of A β tested occurred at or near the same molar ratio of Hsp20-MBP to A β between 0.0005 and 0.001. The molar ratio of Hsp20-MBP to A β needed to optimally prevent fibril formation was several orders of magnitude lower than that needed to prevent aggregation of ADH.

Electron micrographs of A β fibrils, Hsp20-MBP, and A β -Hsp20-MBP mixtures that correspond to conditions used in the Congo Red binding studies are shown in Figure 5. In the images, 100 μ M A β and 0.1 μ M Hsp20-MBP were used. As seen in Figure 5A, A β formed long individual fibrils and groups of long fibrils under the aggregation conditions employed. Hsp20-MBP, at 0.1 μ M and room temperature, formed species of $\sim$ 13.6 nm in diameter, composed of two subunits, 5.4 nm in width each, with a distance of 2.7 nm between subunits (Fig. 5B). In micrographs of the A β -Hsp20-MBP mixture (Fig. 5C), small globular species with a 16.3-nm diameter and variable length were observed. These species could be an Hsp20-A β complex, large Hsp20-MBP aggregates, and/or A β -protofibrils. A β fibrils were noticeably absent from micrographs of A β -Hsp20 mixtures.

Ability of Hsp20-MBP to prevent toxicity of A β

A β is typically toxic to neuron-like cells (SY5Y cells, and PC12 cells etc.) when in aggregated form. In Figure 6, we show the effect of Hsp20-MBP on A β toxicity as measured using the MTT assay for 100 μ M A β added to SH-SY5Y cells and 2 μ M A β added to PC12 cells. Hsp20-MBP by itself had no effect on SH-SY5Y or PC12 cell viability. However, Hsp20-MBP, when added to A β prior to aggregation, had a profound effect on A β toxicity observed in

Figure 6. Relative cell viability of SY5Y cells (triangles) and PC12 cells (circles) treated with 100 μ M (triangles) and 2 μ M (circles) A β as a function of Hsp20-MBP concentration. Viability is measured via the MTT reduction assay. $N \geq 6$. A β (1–40) was incubated for 24 h in the media prior to addition to the cells. Viability as a function of molar ratio of Hsp20-MBP to A β ; viability is reported relative to control cells.

both cell types. MBP, when added to A β , did not attenuate A β toxicity. The toxicity attenuation behavior observed in Figure 6 mirrors the trends in fibril formation prevention seen in Figure 4. The concentration of Hsp20-MBP needed for optimal toxicity prevention appears to scale with A β concentration, with less Hsp20-MBP needed to prevent toxicity when less A β was present. The molar ratio of Hsp20 to A β needed for optimal toxicity attenuation is approximately the same as the molar ratio needed for optimal fibril-formation prevention.

Ability of His-Hsp20 to prevent A β aggregation and toxicity

While control experiments demonstrated that MBP alone had no effect on A β aggregation and toxicity, it was possible that the MBP fusion contributed to the function of Hsp20. To examine this possibility, we next generated an N-terminal polyhistidine-Hsp20 fusion protein. In experiments parallel to those described above, we examined if the polyhistidine-Hsp20 fusion protein could also prevent A β aggregation and toxicity as measured by Congo Red binding and MTT reduction, respectively. As seen in Figure 7, his-Hsp20 prevents A β aggregation and toxicity over a wider

Figure 5. Representative electron micrographs of A β and Hsp20-MBP after 24 h of aggregation (A) 100 μ M A β , (B) 0.1 μ M Hsp20-MBP, and (C) 100 μ M A β + 0.1 μ M Hsp20-MBP.

Figure 7. Relative A β fibril formation and SH-SY5Y cell viability as a function of concentration his-Hsp20. (A) Fibril formation is reported relative to fibril formation in pure 50 mM A β solutions and determined via Congo Red binding. (B) Cell viability was measured via MTT reduction and reported relative to cells untreated with A β or his-Hsp20. A β or his-Hsp20 mixtures were mixed, then aggregated with mixing at 37°C for 24 h prior to viability or Congo Red binding measurements.

range of concentrations and at lower concentrations than the MBP fusion protein. Also, attenuation of toxicity approached 100% with the his-Hsp20 while the Hsp20-MBP attenuated A β toxicity to a lesser extent. However, as with the MBP fusion protein, there was a noticeable loss of His-Hsp20 activity at concentrations above 1 μ M.

Size exclusion chromatography of his-Hsp20

To examine the mechanism of loss of activity of his-Hsp20 at higher concentrations we examined size of his-Hsp20 oligomers in solution as a function of concentration. Stock solutions of his-Hsp20 were diluted to 100 μ M, 29 μ M, and 0.1 μ M in PBS shortly before analysis. Chromatograms of his-Hsp20 at the different concentrations can be seen in Figure 8. At the highest concentration (Fig. 8A), his-Hsp20 elutes as a mixture of a number of oligomers, ranging from an approximate 16-mer, 10-mer, 6-mer, tetramer, dimer, and monomer, with dimer representing about 15% of total protein eluted the column. At intermediate concentrations (Fig. 8B), a mixture of oligomers is still seen, with dimer making up about 42% of the protein eluted from the column. At the lowest concentration, 0.1 μ M, a concentration at which his-Hsp20 displays both high aggregation and toxicity prevention activities, only dimer was observed. Analytical ultracentrifugation was used to confirm the presence of high molecular weight oligomers of his-Hsp20 at high concentrations (data not shown).

Inability of Hsp20-MBP to reverse A β aggregation

We examined if, once A β was aggregated, the addition of Hsp20-MBP could reverse fibril formation. We aggregated A β under conditions analogous to those described previously and, after 24 h aggregation, we added Hsp20 at optimal concentrations for fibril formation prevention determined from earlier experiments (100 nM). At all times measured after addition of Hsp20 to aggregated A β up to 48 h, there was no loss in fibril content as measured by Congo

Figure 8. Representative size exclusion chromatograms of his-Hsp20 at (A) 100 μ M his-Hsp20, (B) 29 μ M his-Hsp20, and (C) 0.1 μ M his-Hsp20. Separations were performed on a Pharmacia Superose 6 column at room temperature using phosphate buffered saline as an elution buffer.

Red binding and no prevention of A β toxicity as measured by the MTT assay in SH-SY5Y cells. Indeed, fibril formation proceeded at the same approximate rate as if no Hsp20 had been added (data not shown).

Discussion

A protein, Hsp20, isolated by Rice-Ficht and coworkers from the bovine erythrocyte parasite *B. bovis* (Brown et al. 2001) has repeatedly demonstrated α -crystallin activity in vitro. In the results presented here, we demonstrate that Hsp20 both reduces A β fibril formation at very low molar ratios, and reduces A β toxicity at similar molar ratios. While other small heat shock proteins and α -crystallins have also been examined for their ability to prevent A β and other amyloid fibril formation with good success at relatively low molar ratios of α -crystallin to amyloid (Kudva et al. 1997; Hughes et al. 1998; Stege et al. 1999; Hatters et al. 2001), in none of these studies has toxicity inhibition been reported. Indeed, in work reported by Stege and coworkers (1999), α -B-crystallin prevented A β fibril formation but enhanced toxicity. Thus, we believe that Hsp20, which has limited sequence homology with other α -crystallins, may act via a unique mechanism to prevent A β aggregation and toxicity, and may provide insight on how to design the next generation of A β aggregation inhibitors to prevent A β toxicity associated with AD.

We show that Hsp20 reduces aggregation of ADH at an optimal mole ratio of 2 Hsp20 to 1 ADH (Figs. 1, 2). This is consistent with the observations of van Montfort et al. (2001) that other alpha crystallins are active as dimers. We show that Hsp20-MBP and his-Hsp20 prevent A β fibril formation as indicated by the absence of Congo Red binding and of visible fibrils in electron micrographs, at molar ratios near 1:1000 Hsp20 to A β or lower (Figs. 3–5, 7). This is in sharp contrast to the near 2:1 molar ratio needed to prevent ADH aggregation. A number of investigators have explored the ability of other small heat shock proteins, molecular chaperones, and α -crystallins to prevent fibril formation in several systems including A β (Kudva et al. 1997; Hughes et al. 1998; Stege et al. 1999; Hatters et al. 2001). Most have found that the chaperones or small heat shock proteins can inhibit A β aggregation at molar ratios of chaperone to A β of 1:10 to 1:100. The low concentrations of Hsp20 and other α -crystallins needed to prevent fibril formation, relative to that needed to prevent ADH aggregation, could be related to specificity of the α -crystallins for β -sheet or fibril-forming proteins. Alternatively, the difference in molar ratios needed to prevent ADH aggregation relative to those needed to prevent A β fibril formation could be related to the temperature differences at which the experiments were carried out. The ADH aggregation experiments were carried out at elevated temperature, while the A β fibril formation experiments were carried out at room temperature. The quaternary

structure of α -crystallins are generally temperature dependent; however, most often, more highly active oligomers (dimers) are formed at higher temperatures (Abgar et al. 2001; van Montfort et al. 2001). Indeed, when we performed fibril formation and toxicity prevention experiments with Hsp20-MBP and A β at 37°C instead of room temperature, ~10 times less Hsp20-MBP was needed to prevent fibril formation (or 1:10,000 Hsp20 to A β mole ratio) relative to that needed to maximally prevent fibril formation at room temperature (data not shown).

Another plausible explanation for Hsp20 activity is that Hsp20 interacts with an oligomer of A β in a near 2:1 molar ratio; however, the A β oligomer may be very dilute in A β solutions. In models of A β aggregation, investigators postulate that A β forms micelles or multimeric nuclei from A β monomers or dimers via a high order process (Lomakin et al. 1996; Pallitto and Murphy 2001; Yong et al. 2002). It is from these micelles or nuclei that fibril growth is initiated. Micelles or nuclei would be far less abundant than monomeric A β . We propose that binding to or removal of these fibril-initiating species from solution would be sufficient to prevent A β fibril formation.

The difference in fibril prevention activity of the two Hsp20 constructs tested, Hsp20-MBP and his-Hsp20, are clearly demonstrated in Figure 7. We speculate that the almost 100-fold increase in effectiveness of the his-Hsp20 compared to the Hsp20-MBP at preventing fibril formation is due to higher affinity of the his-Hsp20 for A β than Hsp20-MBP affinity for A β . Differences in Hsp20 structure or stability in the two different constructs may also affect fibril prevention activity of the protein.

As seen in Figures 4 and 7, there is an optimum concentration of Hsp20 for A β fibril-formation prevention. We suggest several possible explanations for this observation. Oligomerization dynamics of α -crystallins are concentration-dependent (McHaourab et al. 2002). Hsp20 may aggregate to form a less active structure at higher concentrations. High molecular weight oligomers of heat shock proteins, which tend to form at high concentrations, are less active than the low molecular weight oligomers (Liang 2000). Additionally, equilibrium distribution of oligomeric Hsp20 species are likely dependent upon conformers or oligomers of substrate A β present in solution. Size exclusion chromatography (SEC) data presented in Figure 8 supports the hypothesis that Hsp20 oligomerization is concentration dependent. At concentrations where his-Hsp20 has high A β fibril prevention activity, his-Hsp20 elutes as a dimer from the SEC column, suggesting that the active form of his-Hsp20 is a dimer. At concentrations of his-Hsp20 where the protein displays less fibril prevention activity or no activity, complex mixtures of oligomers were detected via both SEC (Fig. 8) and analytical ultracentrifugation (data not shown). These data suggest that oligomers larger than the dimer are inactive. Hsp20-MBP becomes inactive in the fibril forma-

tion assay at lower concentrations than his-Hsp20, suggesting that the MBP construct contributes to the destabilization of the active dimer. Changes in oligomerization of Hsp20-MBP at low concentrations could not be confirmed because of limits of sensitivity of detection of the FPLC system in the range of concentrations where Hsp20-MBP was active.

Analogous to fibril formation prevention data, Hsp20 attenuates A β toxicity at similar molar ratios (Figs. 6, 7). These data suggest that the A β species which bind Congo Red and have fibril appearance in electron micrographs are toxic, or, more probably, A β species which are associated with the formation of Congo Red binding or fibril species are toxic. While nonfibrillar diffusible A β species (sometimes referred to as ADDLs), low molecular weight oligomers, protofibrils, and fibrils have all been found to be toxic (Pike et al. 1993; Lambert et al. 1998; Kaye et al. 2003; and others), recent evidence suggests that oligomeric A β are the toxic (or most toxic) species (Dahlgren et al. 2002; Wang et al. 2002; Kaye et al. 2003; for example).

A careful examination of electron micrographs of A β -Hsp20 mixtures under optimal fibril formation and toxicity prevention conditions (Fig. 5) reveals the presence of small globular species. The globular species are not yet well characterized, however, size analysis suggests that the species may be an Hsp20-A β complex rather than A β protofibrils since the width of these species is atypical for A β micelles (5–11 nm), protofibrils (4–8 nm), or fibrils (6–10 nm) (Yong et al. 2002).

A number of different approaches have been explored for prevention of A β amyloid fibril formation and toxicity. Pentapeptides such as KLVFF (Pallitto et al. 1999), LPFFD (Soto et al. 1998), GVVIN, and RVVIA (Hetenyi et al. 2002) have been used to disrupt A β fibril formation and neurotoxicity. These pentapeptides have the same or similar residues as segments of A β essential for fibril formation, bind to A β , and alter the structure that A β adopts (Soto et al. 1998). Generally, 1:1 molar ratios of peptide inhibitors to A β are needed in order to effectively prevent fibril formation (Pallitto et al. 1999). Amphipathic molecules such as hexadecyl-*N*-methylpiperidinium (HMP) bromide or sulfonated molecules such as Congo Red have also been used to prevent A β fibril formation and toxicity. These aggregation and toxicity inhibitors, while effective, appear to act via a different mechanism than Hsp20.

In summary, we believe that we have found, with Hsp20, a novel protein, which reduces both A β fibril formation and toxicity at very low molar ratios. These properties make Hsp20 unique relative to other A β aggregation inhibitors reported in the literature. Upon further investigation of the mechanism of Hsp20 fibril formation and toxicity prevention, we hope to identify essential interactions between A β and Hsp20 necessary for prevention of the formation of toxic A β species.

Materials and methods

Materials

A β (1–40) was purchased from Biosource International. Cell culture reagents were purchased from GibcoBRL. All other chemicals were obtained from Sigma unless otherwise stated.

Hsp20 preparation

Hsp20 was produced in two forms, as an N-terminal fusion protein with maltose binding protein (Hsp20-MBP) and as an N-terminal polyhistidine fusion protein (his-Hsp20). Both proteins were produced in *Escherichia coli*. The production of the maltose binding protein fusion has been previously described (Brown et al. 2001). The N-terminal polyhistidine fusion protein was made with an intervening Tobacco Etch Virus (TEV) protease site (Carrington et al. 1988) between Hsp20 and a polyhistidine tract. The construct was created through PCR of the Hsp20 coding sequence using a primer containing a 5', 21-bp extension encoding the TEV protease site. The PCR product was introduced into the pTrcHis2 TOPO protein expression vector (Invitrogen) and transformed into chemically competent TOP10 cells (Invitrogen). His-Hsp20 was prepared by growing cells to OD₆₀₀ of 0.5 followed by induction with 1 mM IPTG for 5 h, and removal of media. The cells were then lysed in PBS using a French pressure cell and the insoluble his-Hsp20 pellet collected. The pellet containing his-Hsp20 was suspended in Denaturing Binding Buffer (Invitrogen) containing 8 M urea and incubated with Probond resin for 1 h to allow binding of his-Hsp20 to the nickel chromatography resin. The protein was eluted from the resin using 100 mM EDTA, dialyzed overnight into PBS (pH 7.0), 20% glycerol, and frozen at –80°C. Protein purity and molecular weight were confirmed by SDS PAGE. Upon use, Hsp20 was diluted into buffers that did not contain additional EDTA.

A β peptide preparation

Stock solutions of 10 mg/mL A β (1–40) peptides were dissolved in 0.1% (v/v) trifluoroacetic acid (TFA) in water. After incubation for 20–30 min at 25°C, the peptide stock solutions were diluted to concentrations of 0.5 mg/mL by addition of sterile phosphate buffered saline (PBS; 0.01 M NaH₂PO₄, 0.15 M NaCl, pH 7.4). The peptides were diluted to final concentrations of 20 μ M, 50 μ M, and 100 μ M by addition of PBS for Congo Red and electron microscopy (EM) studies. Cell culture medium (below) replaced PBS in toxicity studies. These peptide solutions were rotated at 60 revolutions per minute at 25°C for 24 h to ensure aggregation. In solutions of peptide alone, evidence of fibril formation as measured by Congo Red binding was seen after a lag period of 4–5 h. Congo Red binding increased rapidly after the lag period, then appeared to approach a constant maximum level at between 24 and 48 h.

Cell culture

Human neuroblastoma SH-SY5Y cells (a gift of Dr. Evelyn Tiffany-Castiglioni, College of Veterinary Medicine, Texas A&M University) were cultured in minimum essential medium (MEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 3 mM L-glutamine, 100 U/mL penicillin, 100 μ g/mL streptomycin, and 2.5 μ g/mL amphotericin B (fungizone) in a humidified 5% (v/v)

CO₂/air environment at 37°C. Rat pheochromocytoma PC12 cells (ATCC) were cultured in RPMI medium supplemented with 10% (v/v) horse serum, 5% (v/v) FBS, 3 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, and 2.5 µg/mL fungizone in a humidified 5% (v/v) CO₂/air environment at 37°C. For the toxicity assays, cells were plated at a density of 1×10^5 cells/well in 96-well plates and aggregated peptide solutions were added to the cells 24 h after plating.

Congo Red binding

Congo Red studies were performed to assess the presence of amyloid fibrils in Aβ solution. Congo Red dye was dissolved in PBS to a final concentration of 120 µM. Congo Red solution was added to the peptide solutions at the ratio of 1:9. The peptide solution and control solution were allowed to interact with Congo Red for 30–40 min prior to absorbance measurement with a Model 420 UV-Vis spectrophotometer (Spectral Instruments) at 25°C. The fibril formation of the samples was estimated from the absorbance using equation 1:

$$[A\beta_{\text{Fib}}] = ({}^{541}A_t/4780) - ({}^{403}A_t/6830) - ({}^{403}A_{\text{CR}}/8620) \quad (1)$$

where $[A\beta_{\text{Fib}}]$ is the concentration of Aβ fibril, ${}^{541}A_t$, ${}^{403}A_t$, and ${}^{403}A_{\text{CR}}$ are the absorbances of the sample and Congo Red at the wavelength of 541 nm and 403 nm, respectively (Klunk et al. 1999). From these data, relative fibril concentrations were calculated as the ratio of sample fibril concentration to pure Aβ fibril concentration.

MTT reduction assay

Cell viability was measured 24 h after peptide addition to cells using the 3, (4,5-dimethylthiazol-2-yl) 2,5-diphenyl-tetrazolium bromide (MTT) reduction assay. Ten microliters of 12 mM MTT was added to 100 µL of cells plus medium in a 96-well plate. The cells were incubated with MTT for 4 h in a CO₂ incubator. Then, 100 µL of a 5:2:3 N, N-dimethylformamide (DMF): sodium dodecyl sulfate (SDS): water solution (pH 4.7) was added to dissolve the formed formazan crystals. After 18 h incubation in a humidified CO₂ incubator, the results were recorded by using an Emax Microplate reader at 585 nm (Molecular Devices). Percentage cell viability was calculated by comparison between the absorbance of the control cells and that of peptide or peptide:Hsp20 treated cells.

Electron microscopy (EM)

Aβ peptide solution (200 µL), prepared as described above, was mixed, placed on glow discharged grids, and then negatively stained with 1% aqueous ammonium molybdate (pH 7.0). Grids were examined in a Zeiss 10C transmission electron microscope at an accelerating voltage of 80 kV. Calibration of magnification was done with a 2,160 lines/mm crossed line grating replica (Electron Microscopy Sciences).

Turbidity assay

ADH turbidity assay

Light Scattering of ADH and Hsp20 was performed as previously described (Horwitz 1992). Briefly, the aggregation of ADH and Hsp20 in solution was measured by the apparent absorption

due to scattering at 360 nm in a Gilford Response II spectrophotometer at 58°C. Solutions were mixed at room temperature in 50 mM phosphate buffer (pH 7.0) and analyzed immediately. In all experiments 1.625 µM ADH was used. Hsp20 concentrations were varied to obtain different molar ratios of Hsp20 to ADH. Absorbance readings were taken at 1-min intervals for 1 h.

Size exclusion chromatography

The size of his-Hsp20 oligomers as a function of concentration at room temperature was determined using size exclusion chromatography using a Pharmacia FPLC system. Protein samples at different concentrations were loaded on a Superose 6 HR 10/300 column (Pharmacia) and eluted using PBS. Eluted proteins were detected using UV absorbance at either 280 nm or 254 nm. Oligomer sizes were estimated based on the elution volumes of a set of calibration proteins run on the same column under similar conditions. In no cases were chelating agents added to the his-Hsp20 solutions in an attempt to prevent aggregation of the his tagged protein.

Statistical analysis

For each experiment, at least three independent determinations were made. Significance of results was determined via the student t test with $p < 0.05$ unless otherwise indicated. Data are plotted as the mean plus or minus the standard error of the measurement.

Acknowledgments

This work was supported by grants from the National Science Foundation and The Welch Foundation to T.G. and by a grant from the National Aeronautics and Space Administration to A.R.-F.

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