

1-1-2010

AlphaB-crystallin inhibits the cell toxicity associated with amyloid fibril formation by kappa-casein and the amyloid-beta peptide

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Recommended Citation

Dehle, Francis C.; Ecroyd, Heath; Musgrave, Ian F.; and Carver, John A.: AlphaB-crystallin inhibits the cell toxicity associated with amyloid fibril formation by kappa-casein and the amyloid-beta peptide 2010, 1013-1026.

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Abstract

Amyloid fibril formation is associated with diseases such as Alzheimer's, Parkinson's, and prion diseases. Inhibition of amyloid fibril formation by molecular chaperone proteins, such as the small heat-shock protein α B-crystallin, may play a protective role in preventing the toxicity associated with this form of protein misfolding. Reduced and carboxymethylated κ -casein (RCM κ -CN), a protein derived from milk, readily and reproducibly forms fibrils at physiological temperature and pH. We investigated the toxicity of fibril formation by RCM κ -CN using neuronal model PC12 cells and determined whether the inhibition of fibril formation altered its cell toxicity. To resolve ambiguities in the literature, we also investigated whether fibril formation by amyloid- β 1-40 (A β 1-40), the peptide associated with Alzheimer's disease, was inhibited by α B-crystallin and if this affected the toxicity of A β . To this end, either RCM κ -CN or A β 1-40 was incubated at neutral pH to induce fibril formation before treating PC12 cells and assessing cell viability. Incubated (fibrillar) RCM κ -CN was more toxic to PC12 cells than native RCM κ -CN with the highest level of toxicity being associated with mature fibrils and protofibrils. Furthermore, the toxicity of RCM κ -CN was attenuated when its fibril formation was inhibited, either through the chaperone action of α B-crystallin or when it interacted with its natural binding partners in milk, α S- and β -casein. Likewise, incubating A β 1-40 with α B-crystallin inhibited both A β 1-40 fibril formation and the associated cell toxicity. Importantly, by inhibiting fibril formation, α B-crystallin prevents the cell toxicity associated with protein misfolding.

Keywords

crystallin, inhibits, cell, toxicity, alphas, beta, kappa, casein, associated, peptide, amyloid, fibril, formation, CMMB

Disciplines

Life Sciences | Physical Sciences and Mathematics | Social and Behavioral Sciences

Publication Details

Dehle, F. C., Ecroyd, H., Musgrave, I. F. & Carver, J. A. (2010). AlphaB-crystallin inhibits the cell toxicity associated with amyloid fibril formation by kappa-casein and the amyloid-beta peptide. *Cell Stress and Chaperones*, 15 (6), 1013-1026.

α B-Crystallin inhibits the cell toxicity associated with amyloid fibril formation by κ -casein and the amyloid- β peptide

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Received: 14 May 2010 / Revised: 24 June 2010 / Accepted: 25 June 2010 / Published online: 15 July 2010
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Abstract Amyloid fibril formation is associated with diseases such as Alzheimer's, Parkinson's, and prion diseases. Inhibition of amyloid fibril formation by molecular chaperone proteins, such as the small heat-shock protein α B-crystallin, may play a protective role in preventing the toxicity associated with this form of protein misfolding. Reduced and carboxymethylated κ -casein (RCM κ -CN), a protein derived from milk, readily and reproducibly forms fibrils at physiological temperature and pH. We investigated the toxicity of fibril formation by RCM κ -CN using neuronal model PC12 cells and determined whether the inhibition of fibril formation altered its cell toxicity. To resolve ambiguities in the literature, we also investigated whether fibril formation by amyloid- β 1–40 (A β _{1–40}), the peptide associated with Alzheimer's disease, was inhibited by α B-crystallin and if this affected the toxicity of A β . To this end, either RCM κ -CN or A β _{1–40} was incubated at neutral pH to induce fibril formation before treating PC12 cells and assessing cell viability. Incubated (fibrillar) RCM κ -CN was more toxic to PC12 cells than native RCM κ -CN with the highest level of toxicity being associated with mature fibrils and protofibrils. Furthermore, the toxicity of RCM κ -CN was

attenuated when its fibril formation was inhibited, either through the chaperone action of α B-crystallin or when it interacted with its natural binding partners in milk, α _S- and β -casein. Likewise, incubating A β _{1–40} with α B-crystallin inhibited both A β _{1–40} fibril formation and the associated cell toxicity. Importantly, by inhibiting fibril formation, α B-crystallin prevents the cell toxicity associated with protein misfolding.

Keywords α B-Crystallin · κ -Casein · Amyloid- β · Amyloid fibril · Cell toxicity · Small heat-shock protein

Abbreviations

A β	amyloid- β
AD	Alzheimer's disease
PD	Parkinson's disease
RCM κ -CN	Reduced and carboxymethylated κ -casein
sHsp	small heat-shock protein
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
TEM	transmission electron microscopy
ThT	Thioflavin T

Introduction

The formation of amyloid fibrils is associated with a diverse array of diseases such as Alzheimer's disease (AD), Parkinson's disease (PD), prion diseases, and type II diabetes (Caughey and Lansbury 2003; Stefani and Dobson 2003; Chiti and Dobson 2006; Roychaudhuri et al. 2009; Yankner and Lu 2009). Amyloid fibrils are self-assembled, ordered aggregates of a normally soluble protein or peptide. The process of amyloid formation arises

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from initial misfolding of a globular protein or the adoption of partial structure by an intrinsically disordered protein or peptide. The resultant partially folded intermediate(s) expose greater hydrophobicity to solution, which can lead to their mutual association, via a nucleation-dependent β -sheet stacking mechanism (Harper and Lansbury 1997; Dobson 2004; Hamley 2007), and the subsequent formation of protofibrils and mature fibrils. Protofibrils are small, soluble oligomeric forms of the protein that are rich in β -sheet and resistant to degradation. Further aggregation of protofibrils results in the formation of mature fibrils, which typically consist of four to six strands in a helical, rope-like structure with its β -sheet backbone orthogonal to the fibril axis.

In cell models of AD, PD, and prion diseases, the amyloidogenic proteins associated with these diseases are toxic in their oligomeric and/or fibrillar form but not in their monomeric state (El-Agnaf et al. 1998; Bodles et al. 2000; Novitskaya et al. 2006; Chimon et al. 2007). Furthermore, fibrillar aggregates of non-disease-related proteins are toxic, which suggests that toxicity is related to the mechanism of fibril formation and/or the overall fibril structure (Bucciantini et al. 2002) rather than the native state of the proteins that form them. Interestingly, evidence suggests that soluble, prefibrillar oligomeric species are at least, if not more, toxic than the mature fibrils, particularly with regard to amyloid- β (A β) peptides, the putative causative agents in AD (Hartley et al. 1999; Caughey and Lansbury 2003; Hoshi et al. 2003; Chimon et al. 2007; Haass and Selkoe 2007). Similar results have also been reported for the putative causative agents of PD and Creutzfeldt–Jakob disease, α -synuclein and the prion protein, respectively, in cell culture models (Du et al. 2003; Simoneau et al. 2007). Therefore, it has been speculated that prefibrillar oligomers, rather than mature fibrils, are responsible for disease progression (Caughey and Lansbury 2003; Haass and Selkoe 2007). By contrast, others have found that mature fibrils of the prion protein are more toxic than protofibrillar aggregates (Novitskaya et al. 2006). Thus, the most toxic species may vary depending on the fibril-forming protein.

Previous research in our laboratory has shown that the reduced and carboxymethylated form of the milk protein, κ -casein (RCM κ -CN), spontaneously forms fibrils in a highly reproducible manner over a 10–15 h timeframe when incubated under conditions of physiological temperature and pH, without the need for denaturants (Thorn et al. 2005). These properties of RCM κ -CN, coupled with its ready availability, make it a very useful model to study the process of fibril formation (Ecroyd et al. 2008) and to screen for fibril-inhibiting compounds in a high throughput manner (Carver et al. 2010).

In milk, κ -casein is present in a micelle-like structure along with the other casein proteins, α _S-casein (which is

comprised of the unrelated α _{S1}- and α _{S2}-casein proteins) and β -casein. The primary function of these micelles is believed to be their nutritional benefit through their role in transporting calcium (Fox and McSweeney 1998). We have recently shown that the interaction and aggregation of the casein proteins also prevents fibril formation by the amyloidogenic κ - and α _{S2}-caseins (e.g., with β - and α _{S1}-casein, respectively; Thorn et al. 2005, 2008). RCM κ -CN fibril formation is potently inhibited by α _S- and β -casein *in vitro* (Thorn et al. 2005). α _S- and β -casein have structural and functional properties similar to the small heat-shock family of molecular chaperone proteins (sHsps) by inhibiting the ordered and disordered forms of protein aggregation in a chaperone-like manner (Morgan et al. 2005; Thorn et al. 2005, 2008, 2009). *In vivo*, this is physiologically relevant since amyloid-like deposits have been found in bovine mammary tissue within calcified stones in corpora amylacea, a disease which blocks the small milk ducts and reduces milk secretion and flow (Reid 1972; Beems et al. 1978; Nickerson et al. 1985; Taniyama et al. 2000). We have proposed that the amyloid deposits in corpora amylacea are caused by κ - and α _{S2}-casein fibril formation due to a reduced chaperone action of the other casein proteins (Thorn et al. 2005, 2008).

The sHsp family of molecular chaperones functions intracellularly by interacting with and stabilizing partially folded protein intermediates to prevent their aggregation, particularly under stress conditions (e.g., elevated temperature; Carver et al. 2003; Ecroyd and Carver 2009). In doing so, sHsps may play a protective role in preventing the misfolding and subsequent fibril formation of amyloidogenic proteins. The ubiquitously expressed α B-crystallin is the most widely studied sHsp and has been shown to inhibit fibril formation by a range of amyloidogenic proteins and peptides *in vitro*, including A β , α -synuclein, RCM κ -CN, apolipoprotein C-II, and ataxin-3 (Hatters et al. 2001; Rekas et al. 2004, 2007; Raman et al. 2005; Wilhelmus et al. 2006a, b; Ecroyd et al. 2007; Robertson et al. 2010). The interaction of α B-crystallin with A β has received the most attention but has produced contrasting results. Stege et al. (1999) and Liang (2000) found that α B-crystallin enhanced β -sheet structure in A β . Furthermore, Stege et al. (1999) observed that α B-crystallin inhibited the formation of mature A β fibrils and so concluded that α B-crystallin stabilizes A β into a more toxic β -sheet-rich oligomeric form. In support of this, α B-crystallin enhanced the toxicity of A β in cultured rat neurons (Stege et al. 1999). In contrast, Raman et al. (2005) and Wilhelmus et al. (2006a, b) reported that α B-crystallin inhibited fibril formation by A β . Wilhelmus et al. (2006a, b) also found that α B-crystallin inhibited the toxicity of A β in human brain pericytes. Likewise, the related sHsp, α A-crystallin, inhibited fibril formation by A β and the associated cell toxicity (Santhoshkumar and Sharma 2004).

Given the proposed common mechanism of toxicity of fibril-forming proteins (Bucciantini et al. 2002), we investigated whether RCM κ -CN is also a suitable model to study the toxicity of fibril formation. Furthermore, in light of the discrepancy in the literature, we sought to determine whether α B-crystallin is a generic inhibitor of amyloid fibril formation and the cell toxicity associated with this process. Similar to other fibril-forming proteins, RCM κ -CN was more toxic to PC12 cells in its fibrillar and prefibrillar forms compared with the native form of the protein. In addition, pre-incubating α B-crystallin with A β and RCM κ -CN inhibited both fibril formation and cell toxicity. Finally, RCM κ -CN's natural binding partners in milk micelles, α _S- and β -casein, also inhibited its amyloid fibril formation and cell toxicity. Our results highlight the importance of α B-crystallin and α _S- and β -casein in preventing fibril formation associated with AD and corpora amylacea, respectively. Moreover, by preventing fibril formation of different fibril-forming proteins, molecular chaperones inhibit the general mechanism of toxicity associated with this process.

Materials and methods

Materials

Bovine α _S-, β -, and κ -casein were purchased from Sigma Chemical Co. (St Louis, MO, USA). Prior to use, the κ -casein was reduced and carboxymethylated as described previously (Farrell et al. 2003). α B-Crystallin was expressed and purified as described previously (Horwitz et al. 1998; Aquilina et al. 2004). The concentration of each protein was determined by spectrophotometric methods using a Cary 5000 UV-visible spectrophotometer (Varian Inc, Palo Alto, CA, USA), and molar absorption coefficients were calculated based on amino acid sequences. A β ₁₋₄₀ was purchased from Peptide Institute Inc. (Osaka, Japan). Uranyl acetate was purchased from Agar Scientific (Stansted, UK). RPMI 1640 powder, fetal bovine serum, horse serum, and L-glutamine were purchased from Thermo Electron Corporation (Victoria, Australia). Penicillin/streptomycin solution and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Co.

PC12 cell culture

PC12 cells were grown in RPMI 1640 medium supplemented with 10% horse serum, 5% fetal bovine serum, 10 U/ml penicillin, and 10 μ g/ml streptomycin. Cells were cultured in uncoated 75 cm² plastic flasks in an incubator with 95% air and 5% CO₂ at 37°C. The medium was

refreshed every 2–3 days. The day prior to treatment, the cells were plated in to 96-well plates at a density of 2×10^4 cells per well in full-serum media.

Cellular toxicity of RCM κ -casein

RCM κ -CN (500 μ M) was dissolved in phosphate buffer (50 mM, pH 7.4). To determine the concentration-response of RCM κ -CN-induced cell toxicity, RCM κ -CN was incubated at 37°C for 20 h to induce fibril formation. Prior to incubation, samples of native RCM κ -CN (non-fibrillated) were taken and stored at -20°C . We found no difference in the toxicity of native or incubated samples that were frozen compared to those that were not frozen (data not shown). Following the 20-h incubation, both the native and fibrillar RCM κ -CN were diluted further in phosphate buffer and added to the cells at the indicated concentrations. The RCM κ -CN-treated cells were then incubated for 48 h followed by an MTT assay (details below) to assess cell viability.

To determine whether the toxicity of species formed during RCM κ -CN fibril formation is time-dependent, the protein (125 μ M) was incubated under the same conditions as described above, and samples of RCM κ -CN were taken after 0, 0.5, 1, 2, 5, and 20 h of incubation and stored at -20°C . Later, these aliquots of RCM κ -CN from the various time points were thawed and added to the cells so that the final concentration of protein was 0.5 μ M. The cells were incubated for 48 h followed by an MTT assay.

In some experiments, after the 20-h incubation, samples of RCM κ -CN were ultracentrifuged at 90,000 rpm (316,613 rcf) for 1 h to separate fibrillar (pellet) and non-fibrillar (supernatant) species of RCM κ -CN. Following centrifugation, the supernatant containing the non-fibrillar RCM κ -CN was collected and the fibrillar RCM κ -CN pellet resuspended in an equal volume of phosphate buffer. The cells were treated with equal volumes of the pellet and supernatant samples.

To test the effect of molecular chaperones on RCM κ -CN-induced cell toxicity, RCM κ -CN (50 μ M) was incubated for 20 h in the presence of inhibitors of RCM κ -CN fibril formation, i.e., α B-crystallin, α _S-casein, and β -casein. α B-Crystallin was incubated at 0.1:1.0, 0.5:1.0, 1.0:1.0, and 2.0:1.0 mol/mol ratio of RCM κ -CN, and α _S- and β -casein was incubated at 0.4:1.0, 0.8:1.0 and 1.6:1.0 mol/mol ratio of RCM κ -CN. Following incubation, the samples were added to the cells so that the final concentration of RCM κ -CN was 0.5 μ M.

Cellular toxicity of A β

A β ₁₋₄₀ (3.8 mM) was dissolved in ammonium hydroxide (1%) and then diluted to 500 μ M in water and stored at

–80°C. Further dilutions were made in 50 mM phosphate and 100 mM NaCl buffer (pH 7.4). To test the effect of α B-crystallin on $A\beta_{1-40}$ -induced cell toxicity, $A\beta_{1-40}$ (10 μ M) was incubated at 37°C for 72 h in the presence of α B-crystallin at 0.01:1.0, 0.1:1.0, and 1.0:1.0 mol/mol ratio of $A\beta_{1-40}$. Samples of $A\beta_{1-40}$ were taken after 0, 24, 48, and 72 h incubation and stored at –20°C. Following incubation, the samples were added to the cells so that the final concentration of $A\beta_{1-40}$ was 0.5 μ M. The cells were incubated for 48 h and cell viability was assessed via an MTT assay.

MTT assay

The MTT assay measures a cell's ability to reduce yellow MTT to purple formazan. This reaction is catalyzed by the mitochondrial enzyme, succinate dehydrogenase. Therefore, the MTT assay is a measure of mitochondrial activity and thus, cell viability. After incubation of the treated cells for 48 h, the media was removed, and 100 μ l of serum-free media containing MTT (0.25 mg/ml) was added to each well. The cells were then incubated for an additional 2 h at 37°C, the media removed and 100 μ l of DMSO added to each well. Absorbance was measured at 570 nm on a BMG Polarstar microplate reader (BMG Labtechnologies, Offenburg, Germany).

Hoechst staining

PC12 cells were plated on to poly-L-lysine (10 μ g/ml) coated coverslips in 6-well culture dishes at a density of 2×10^5 cells per well in full-serum medium and incubated at 37°C for 24 h. The cells were then treated with native and fibrillar RCM κ -CN (0.5 μ M) for 48 h. After the incubation period, the media was removed by aspiration, and the cells were washed several times in cold PBS (pH 7.4). The cells were fixed with 3.7% formaldehyde, permeabilized in 0.1% triton-X 100 and stained with Hoechst 33258 (2 μ g/ml) for 20 min in the dark at room temperature. The coverslips were mounted on glass slides using 90% glycerol. Cells were viewed and recorded using a fluorescence microscope (BX50, Olympus) with a 100 \times objective. Images were recorded using a SPOT camera and a computer with SPOT image analysis software (Diagnostic Instruments, Inc. NSW, Australia) and analyzed using NIH imaging software (National Institutes of Health, USA).

Thioflavin T assay

In situ thioflavin T (ThT) assays were used to monitor the time course of fibril formation by RCM κ -CN and $A\beta_{1-40}$

and to monitor the effect of molecular chaperones on fibril formation. The proteins were incubated at 37°C for 20 h in phosphate buffer (50 mM, pH 7.4) with 10 μ M ThT in black μ Clear 96-microwell plates. Fluorescence was measured with a 440 nm/490 nm excitation/emission filter set on a Fluostar Optima plate reader (BMG Labtechnologies, Offenburg, Germany). ThT fluorescence readings are affected by temperature and an initial decrease in ThT fluorescence is observed as the solutions are heated from room temperature to 37°C (Thorn et al. 2005; Ecroyd et al. 2008). Therefore, the change in ThT fluorescence was measured after this initial decrease.

Transmission electron microscopy

Samples from the above experiments were prepared for transmission electron microscopy (TEM) by adding 2 μ l of protein solution to Formvar and carbon-coated nickel grids (SPI Supplies, West Chester, PA). The grids were then washed three times with 10 μ l of water and negatively stained with 10 μ l of uranyl acetate (2% w/v). Samples were viewed using a Philips CM100 transmission electron microscope (Philips, Eindhoven, The Netherlands).

Statistics

One-way analysis of variance was performed with Bonferroni's and Dunnett's post hoc tests depending on experimental design.

Results

The fibrillar state of RCM κ -CN is cytotoxic to PC12 cells

Previous research has shown that amyloid fibril-forming proteins are more toxic in an aggregated, fibrillar state compared to their native, non-fibrillar form (El-Agnaf et al. 1998; Bodles et al. 2000; Bucciantini et al. 2002; Novitskaya et al. 2006; Chimon et al. 2007). To determine whether this was also the case for RCM κ -CN, PC12 cells were treated with RCM κ -CN in its native state and following incubation in phosphate buffer (50 mM, pH 7.4) at 37°C for 20 h (Thorn et al. 2005; Ecroyd et al. 2007, 2008), conditions under which it forms fibrils (i.e., fibrillar RCM κ -CN). Both native and fibrillar RCM κ -CN caused a concentration-dependent decrease in PC12 cell survival (Fig. 1). However, fibrillar RCM κ -CN was much more toxic at lower concentrations compared with native RCM κ -CN. At 0.5 μ M, fibrillar RCM κ -CN reduced cell viability to $66 \pm 3\%$ compared with $90 \pm 4\%$ for native

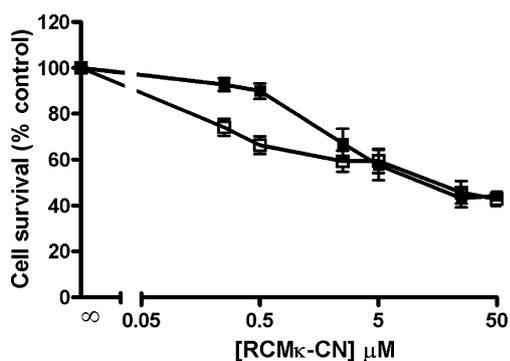


Fig. 1 Concentration-dependent toxicity of native and fibrillar RCM κ -CN. RCM κ -CN (500 μ M) was dissolved in phosphate buffer (50 mM, pH 7.4) and either frozen in its native form or incubated for 20 h at 37°C to induce fibril formation. Native (filled square) and fibrillar (square) forms of RCM κ -CN (0–50 μ M) were then added to the cell culture media of the PC12 cells and the cells incubated for 48 h. Cell survival was assessed using the MTT assay. Values are presented as percentage of cell survival compared with control \pm SEM ($n=6$)

RCM κ -CN. Pre-formed RCM κ -CN fibrils, in the presence of cell media, remain in a fibrillar state for at least 48 h as indicated by ThT fluorescence and TEM images showing the extensive presence of mature fibrils (data not shown). Furthermore, fibril formation by RCM κ -CN still occurs, albeit at a reduced rate, when it is incubated in cell media compared with phosphate buffer (data not shown). Therefore, these results imply that the cell toxicity of fibrillar RCM κ -CN is due to the presence of pre-formed fibrils. Furthermore, the toxicity of native RCM κ -casein most likely arises from fibrillar species that are present in significant quantity due to the marked aggregation propensity of RCM κ -casein which lacks a lag phase in its aggregation profile (Thorn et al. 2005; Ecroyd et al. 2008; Carver et al. 2010). Thus at 0.5 μ M, the toxicity of RCM κ -CN can be attributed to its fibrillar structure. Accordingly, this concentration was used for subsequent experiments.

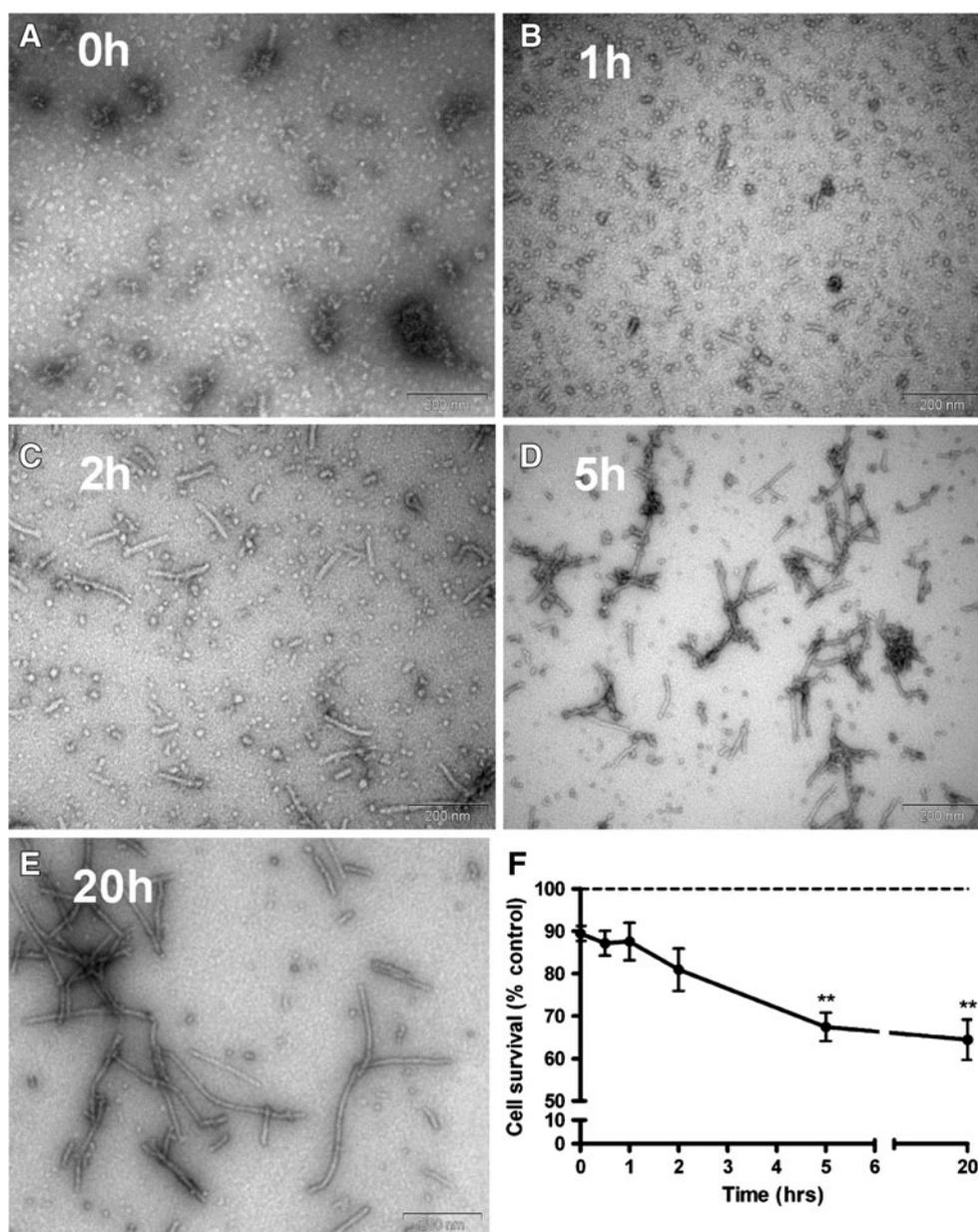
The prefibrillar oligomers and protofibrils of some amyloid fibril-forming proteins, both disease related and unrelated, have previously been shown to be more toxic than, or as toxic as, mature fibrils (Bucciantini et al. 2002; Du et al. 2003; Chimon et al. 2007; Simoneau et al. 2007). By contrast, others have reported that mature fibrils are the most cytotoxic species (Novitskaya et al. 2006). To address the species responsible for the toxicity associated with RCM κ -CN fibril formation, RCM κ -CN was pre-incubated in phosphate buffer for specific time periods and aliquots were taken at different stages of the fibril-forming process. The TEM images (Fig. 2a–e) confirmed that native RCM κ -CN (0 h) consists of primarily spherical, oligomeric micelle-like species (Fig. 2a; Vreeman et al. 1981; Thorn et al. 2005). Similar species were present after 1 h of

incubation (Fig. 2b); however, after 2 h of incubation, both spherical oligomers and protofibril/short fibrillar structures were observed (Fig. 2c). After 5 h of incubation, there was a mixture of protofibrils and mature fibrils evident (Fig. 2d), and after 20 h, more mature fibrils were observed (Fig. 2e). RCM κ -CN incubated for 1 h and 2 h did not significantly affect cell viability compared with the native state of the protein (Fig. 2f); however, after 5 h of incubation, there was a significant decrease in cell survival compared with native RCM κ -CN. This level of toxicity was maintained in the 20-h sample. These results indicate that the toxicity of RCM κ -CN is dependent on the species formed during the fibril-forming process.

The TEM images indicated that not all of the RCM κ -CN had formed fibrils after 20 h (Fig. 2e), which raised the possibility that the cell toxicity of the 20-h sample was due to smaller oligomeric species present rather than the mature fibrils and protofibrils. To determine whether the toxicity of the 20-h sample was associated with the presence of small RCM κ -CN oligomers, the 20-h sample was ultracentrifuged, and mature fibrils and protofibrils were separated from the smaller oligomeric RCM κ -CN species. TEM images indicated that following ultracentrifugation, the pellet contained a mixture of mature fibrils and smaller protofibrillar species (Fig. 3c) whereas the supernatant contained predominately small β -sheet-rich oligomers with a few short fibrils. The species present in the supernatant were rich in β -sheet as indicated by their ability to bind ThT (Fig. 3b). The species present in the pellet were significantly more cytotoxic compared with native RCM κ -CN and had the same level of toxicity as the 20-h sample that had not been ultracentrifuged (Fig. 3a). In contrast, the supernatant sample was no more cytotoxic than native RCM κ -CN. Combined with the results presented in Fig. 2, this suggests that mature RCM κ -CN fibrils and protofibrils are more toxic than the smaller low molecular weight prefibrillar oligomers.

Previous studies have shown that amyloid fibrils cause PC12 cell death via apoptosis (Troy et al. 2000, 2001; O'Donovan et al. 2001; Tanaka et al. 2001; Onoue et al. 2002; Danzer et al. 2007). Therefore, in order to determine whether RCM κ -CN fibrils also cause cell death via the apoptosis pathway, we stained PC12 cells with the Hoechst DNA stain following treatment with native and fibrillar RCM κ -CN. The images show that more cells treated with fibrillar RCM κ -CN have granular staining (Fig. 4), which indicates the condensed chromatin typical of cells undergoing apoptosis. Thus, cells treated with fibrillar RCM κ -CN exhibited granular staining characteristic of late-stage apoptosis (top arrow in Fig. 4b) and chromatin clumping typical of early stage apoptosis (bottom two arrows in Fig. 4b).

Fig. 2 Time-dependent toxicity of RCM κ -CN. **a–e** Electron micrographs of RCM κ -CN (125 μ M) after **a** 0 h, **b** 1 h, **c** 2 h, **d** 5 h, and **e** 20 h incubation at 37°C, showing fibril formation. Scale bar, 200 nm. **f** RCM κ -CN (125 μ M) was dissolved in phosphate buffer (50 mM, pH 7.4) and incubated at 37°C to induce fibril formation. Samples were taken over 0 to 20 h incubation and added to the cell culture media of the PC12 cells (0.5 μ M final concentration). The cells were incubated for 48 h followed by an MTT assay to assess cell survival. Values are presented as percentage of cell survival compared with control \pm SEM. ** p < 0.01 compared with 0 h incubation ($n=4$, one-way ANOVA, Dunnett's test)



Inhibition of fibril formation by RCM κ -CN leads to a corresponding inhibition of the cytotoxicity associated with this process

We have previously shown that α B-crystallin inhibits RCM κ -CN fibril formation (Ecroyd et al. 2007; Rekas et al. 2007). In this study, we extended this finding to determine whether α B-crystallin also affected the cytotoxicity associated with this process. α B-Crystallin inhibited ThT fluorescence in a concentration-dependent manner consistent with previous findings (Fig. 5a; Rekas et al. 2007), and this correlated with an inhibition of the toxicity

of RCM κ -CN to PC12 cells (Fig. 5b). These results indicate that by inhibiting RCM κ -CN fibril formation, α B-crystallin protects against fibril-induced cell toxicity.

In addition, both α S- and β -casein, the natural binding partners of κ -casein in milk micelles, completely inhibited RCM κ -CN fibril formation at a 1.6:1.0 and 0.8:1.0 mol/mol ratio, consistent with our previous findings (Fig. 6a; Thorn et al. 2005). At these concentrations, α S- and β -casein also significantly inhibited the cytotoxicity associated with fibril formation by RCM κ -CN (Fig. 6b). At a 0.4:1.0 mol/mol ratio, α S- and β -casein inhibited ThT fluorescence by 90% and 80%, respectively. However, there was still significant

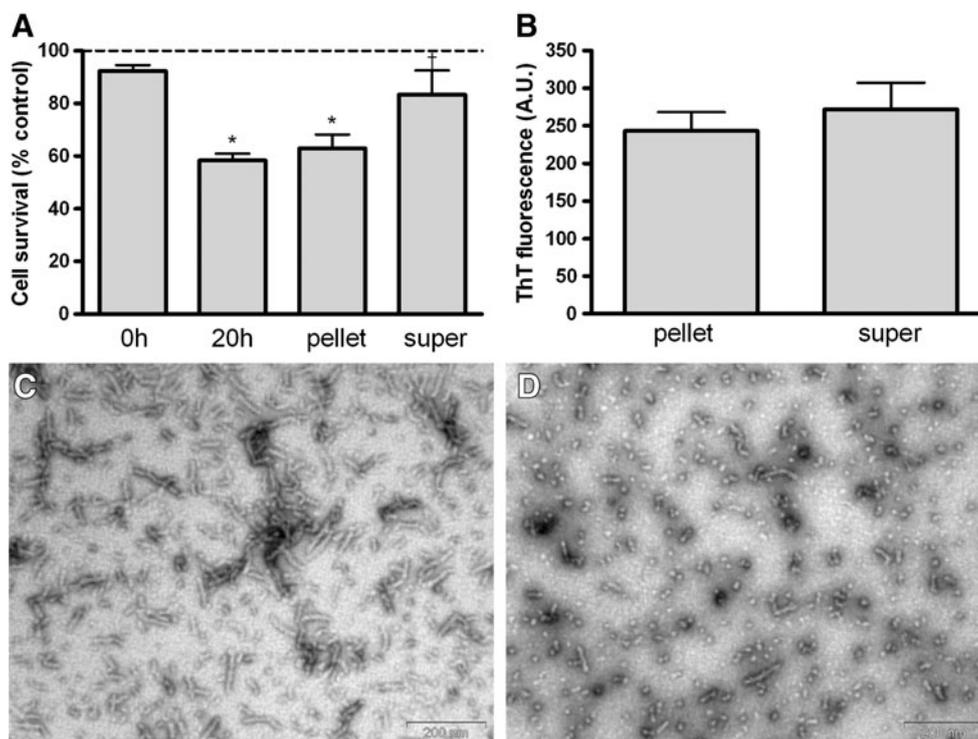


Fig. 3 Analysis of RCMκ-CN following ultracentrifugation. **a** RCMκ-CN (125 μM) was dissolved in phosphate buffer (50 mM, pH 7.4) and incubated for 20 h at 37°C to induce fibril formation. The sample was then ultracentrifuged at 90,000 rpm, supernatant removed, and the pellet resuspended in an equal volume of phosphate buffer. An equal aliquot of the supernatant and pellet was added to the PC12 cells, and the cells were incubated for 48 h followed by an MTT assay

to assess cell survival. Values are presented as percentage of cell survival compared with control±SEM. **p*<0.05 compared with 0 h incubation. *Dagger sign*, *p*<0.05 compared with 20 h incubation (*n*=6, one-way ANOVA, Bonferroni's test). **b** ThT fluorescence of pelleted RCMκ-CN and resulting supernatant. **c, d** Electron micrographs of **c** pelleted RCMκ-CN and **d** resulting supernatant. Scale bar, 200 nm

RCMκ-CN toxicity present at this ratio, implying that there were sufficient species present under these conditions to cause cytotoxicity. Thus, while α_S- and β-casein inhibit fibril formation at low concentrations, complete inhibition is required before their beneficial effects against fibril-associated cell toxicity are observed.

Effect of αB-crystallin on the cytotoxicity associated with fibril formation by Aβ₁₋₄₀

We examined whether the ability of αB-crystallin to prevent the toxicity associated with fibril formation extends to a disease-relevant amyloidogenic species, namely the

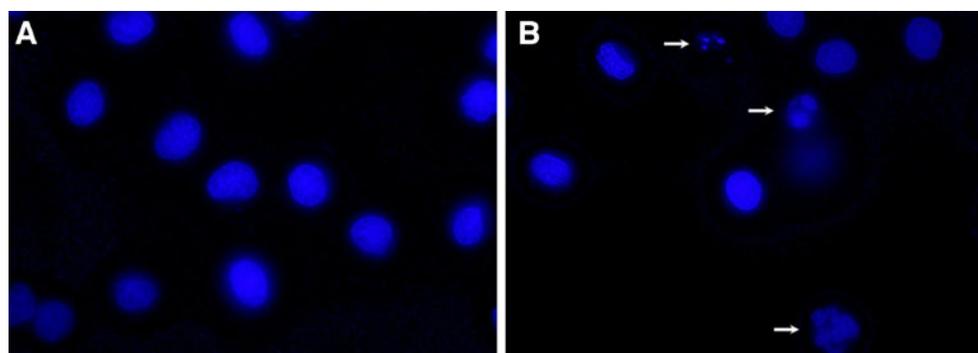


Fig. 4 Hoechst staining of PC12 cells treated with native and fibrillar RCMκ-CN. RCMκ-CN (500 μM) was dissolved in phosphate buffer (50 mM, pH 7.4) and either frozen in its native form or incubated for 20 h at 37°C to induce fibril formation. **a** Native or **b** fibrillar forms of

RCMκ-CN (0.5 μM) were then added to the cell culture media of the PC12 cells, the cells incubated for 48 h, and then stained with Hoechst 33258. Arrows indicate condensed or fragmented nuclei

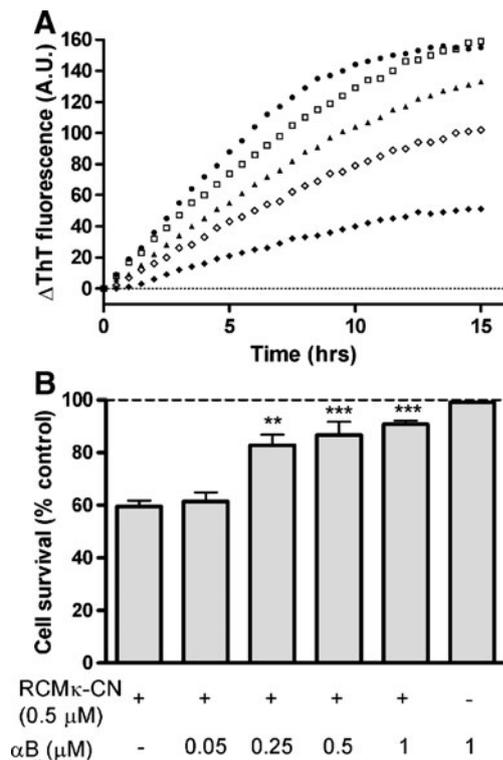


Fig. 5 Effect of α B-crystallin on RCM κ -CN fibril formation and toxicity. **a** RCM κ -CN (50 μ M) was dissolved in phosphate buffer (50 mM, pH 7.4) and incubated for 20 h at 37°C in the absence (filled circle) or presence of α B-crystallin at 5 μ M (square), 25 μ M (filled triangle), 50 μ M (diamond), and 100 μ M (filled diamond); and fibril formation was measured by in situ ThT fluorescence. **b** Samples from the ThT assay were added to the PC12 cells so that the final concentration of RCM κ -CN was 0.5 μ M. The cells were incubated for 48 h followed by an MTT assay to assess cell survival. Values are presented as percentage of cell survival compared with control \pm SEM. ** $p < 0.01$, *** $p < 0.001$ compared with 20 h RCM κ -CN ($n = 3$, one-way ANOVA, Dunnett's test)

$A\beta_{1-40}$ peptide. Previous studies in this area have produced conflicting results (Stege et al. 1999; Wilhelmus et al. 2006a, b). In particular, it has been proposed that α B-crystallin stabilizes a toxic form of $A\beta_{1-40}$ during its fibril-forming process (Stege et al. 1999). However, in these studies, native $A\beta$ and α B-crystallin were applied directly to the cell culture media and the inhibition of fibril formation by α B-crystallin in this media was not established. In the study herein, we sought to definitively establish the effect of α B-crystallin on the cytotoxicity associated with fibril formation by $A\beta$. To this end, we pre-incubated $A\beta_{1-40}$ in the absence or presence of α B-crystallin before exposure to PC12 cells. $A\beta_{1-40}$ formed mature fibrils over 48–72 h as indicated by TEM and ThT fluorescence (Fig. 7a–d). At 0 h, there was some oligomeric $A\beta_{1-40}$ present (Fig. 7a). Larger oligomeric species were observed after 24 h (Fig. 7b), and mature fibrils were observed after 48 and 72 h (Fig. 7c, d). When monitored by in situ ThT fluorescence, the formation of $A\beta_{1-40}$ fibrils

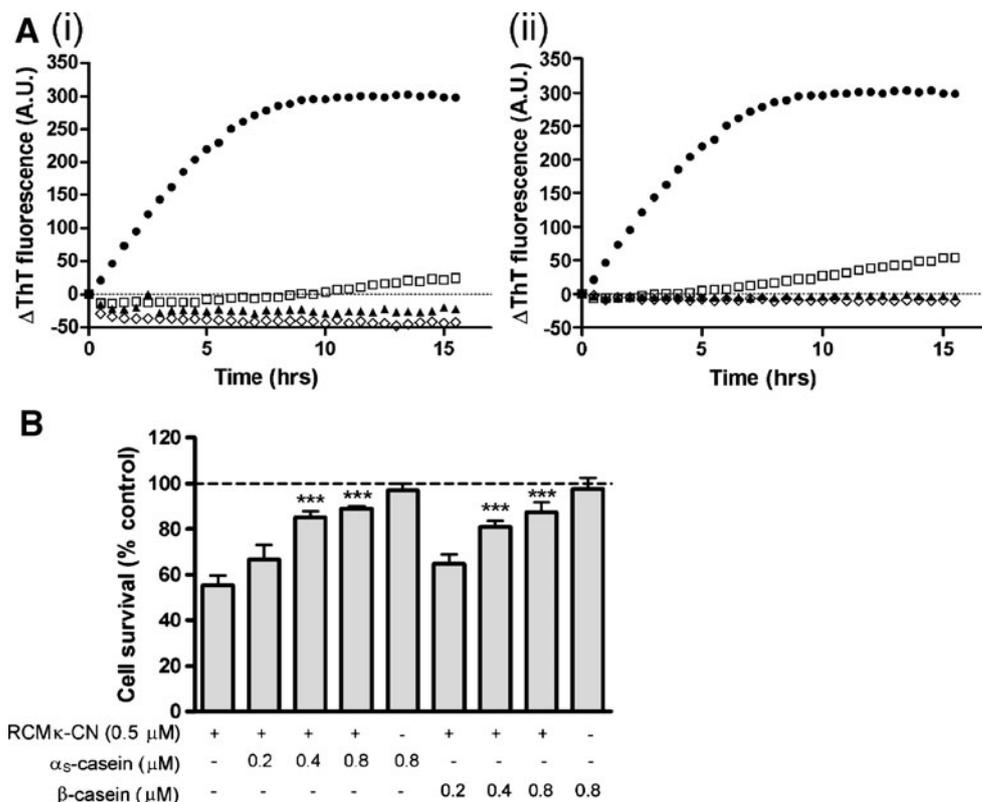
included a long lag phase of approximately 20 h followed by a growth phase, which reached a plateau at approximately 72 h (Fig. 8a). In a similar manner to RCM κ -CN, the toxicity of $A\beta_{1-40}$ was time-dependent, with $A\beta_{1-40}$ fibrils formed after 48 and 72 h producing a significant decrease in cell survival compared with native $A\beta_{1-40}$, whereas smaller aggregates, formed after 24 h, decreased cell survival but this was not found to be significant (Fig. 7e). α B-Crystallin completely inhibited $A\beta_{1-40}$ fibril formation at a 1:100 mol/mol ratio as indicated by the inhibition of ThT fluorescence and the absence of fibrils when viewed by TEM (Fig. 8a, b). Significantly, when the samples of $A\beta_{1-40}$ incubated with α B-crystallin were added to PC12 cells, there was a significant decrease in the cell toxicity of $A\beta_{1-40}$ (Fig. 8c). Together, these results show that inhibition of $A\beta_{1-40}$ amyloid fibril formation by α B-crystallin results in an abolishment of the cell toxicity associated with this process and therefore definitively establishes that the species formed during the interaction between these two proteins is not cytotoxic to cells.

Discussion

We (Thorn et al. 2005, 2009; Ecroyd et al. 2007, 2008) and others (Farrell et al. 2003) have shown previously that RCM κ -CN rapidly forms amyloid fibrils under conditions of physiological temperature and pH via a nucleation-dependent mechanism. For the present study, we have extended these findings to show that fibrils formed by this protein are toxic to PC12 cells (Fig. 1). Our results are consistent with the hypothesis that the toxicity of amyloid fibril-forming proteins is inherent in the mechanism of fibril formation and/or the amyloid fibril structure and that there is a common mechanism of toxicity for amyloid-forming proteins (Bucciantini et al. 2002). Furthermore, as RCM κ -CN forms fibrils under physiological conditions in a relatively short timeframe and without the need for denaturants, it is a good model to study the fibril-forming process and its associated cell toxicity.

Previous cell culture studies have shown that prefibrillar oligomers, protofibrils, and mature fibrils of fibril-forming proteins are toxic, although the relative level of toxicity between these species appears to be dependent on the specific fibril-forming protein. For instance, prion protein oligomers were highly toxic to cortical neurons whereas mature prion protein fibrils demonstrated very little toxicity (Simoneau et al. 2007). However, in a similar study in SH-SY5Y cells and hippocampal neurons, mature prion protein fibrils were as toxic as smaller oligomeric species (Novitskaya et al. 2006). For $A\beta$, β -sheet-rich oligomers, protofibrils, and mature fibrils were toxic (Hartley et al. 1999; Hoshi et al. 2003; Chimon et al. 2007), and in a study

Fig. 6 Effect of α _S-casein and β -casein on RCM κ -CN fibril formation and toxicity. **a** RCM κ -CN (50 μ M) was dissolved in phosphate buffer (50 mM, pH 7.4) and incubated for 20 h at 37°C in the absence (filled circle) or presence of either α _S-casein (i) or β -casein (ii) at 20 μ M (square), 40 μ M (filled triangle), and 80 μ M (diamond); and fibril formation was measured by in situ ThT fluorescence. **b** Samples from the ThT assay were added to the PC12 cells so that the final concentration of RCM κ -CN was 0.5 μ M. The cells were incubated for 48 h followed by an MTT assay to assess cell survival. Values are presented as percentage of cell survival compared with control \pm SEM. ****p* < 0.001 compared with 20 h RCM κ -CN (*n* = 3, one-way ANOVA, Dunnett's test)

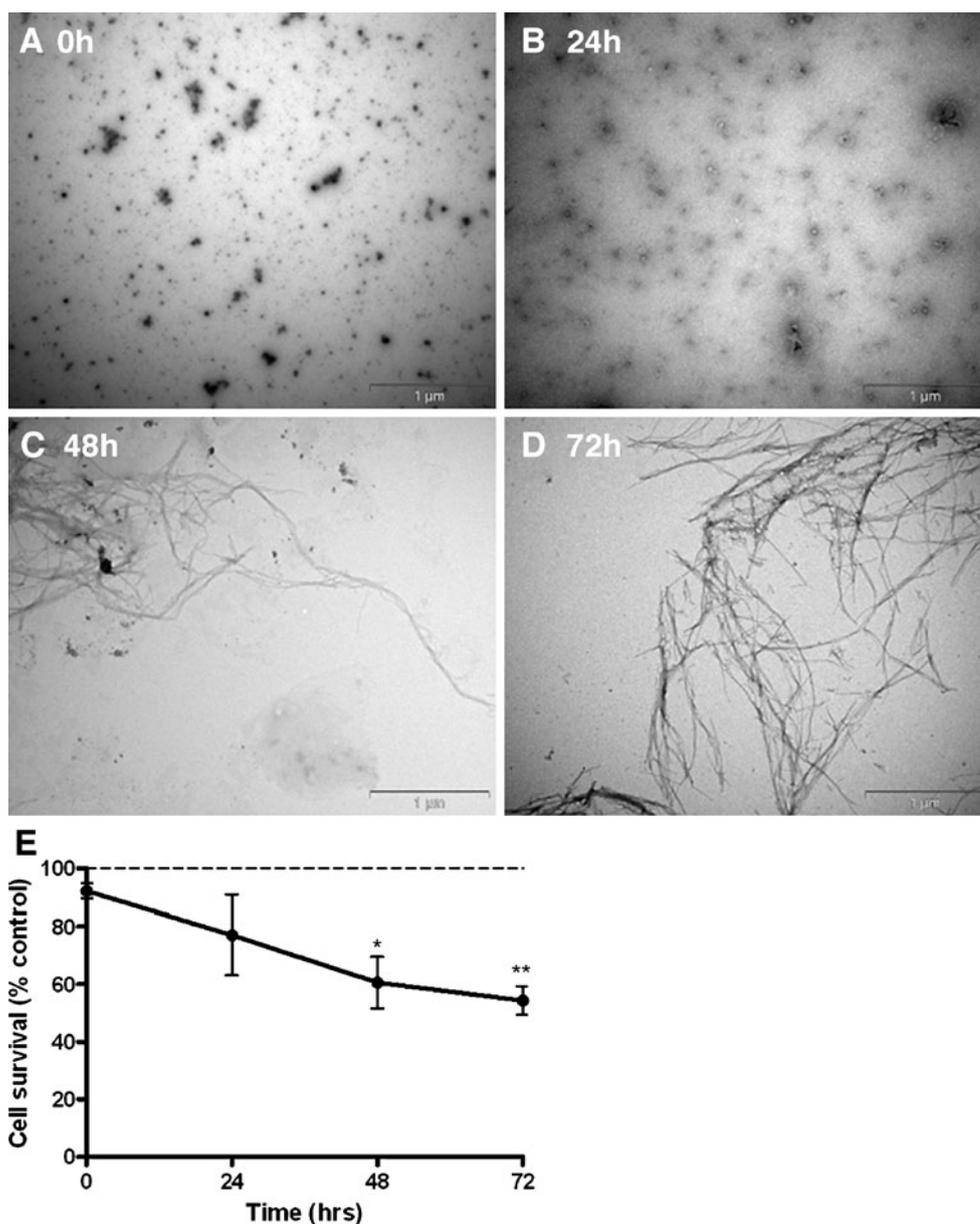


on fibril formation and cell toxicity of non-disease-related proteins, prefibrillar and protofibrillar aggregates were cytotoxic whereas the mature fibrils exhibited no significant toxicity (Bucciantini et al. 2002). Our research indicates that RCM κ -CN fibrils and protofibrils are more toxic than small molecular weight prefibrillar oligomers and the native state of the protein. Thus, after 5 h of incubation, RCM κ -CN formed a mixture of protofibrils and mature fibrils that was significantly more toxic to cells compared with the smaller prefibrillar oligomers formed following 2 h of incubation (Fig. 2). Similar results were observed for A β ₁₋₄₀, where the species formed after 48 and 72 h of incubation were significantly cytotoxic compared with native A β ₁₋₄₀, whereas the species present following 24 h of incubation was not (Fig. 7). It is worth noting that while our data suggest that the mature fibrils formed by both RCM κ -CN and A β ₁₋₄₀ are toxic to neuronal cells, we observed similar levels of cytotoxicity at time points where the ThT fluorescence was not maximal (i.e., 5 h for RCM κ -CN and 48 h for A β ₁₋₄₀). Thus, from these data, we conclude that protofibrillar species, on the pathway to forming mature fibrils and therefore more abundant in these earlier time point samples, appear to be as toxic to neuronal cells as the mature fibrils.

To investigate this further with regard to RCM κ -CN, we ultracentrifuged the sample that had been incubated for 20 h in order to separate the low molecular weight species in the supernatant (native state and β -sheet-rich prefibrillar oligomers) from the high molecular weight species in the

pellet (protofibrils and mature fibrils). We found that the species present in the supernatant did not significantly affect cell survival, having the same level of toxicity as the native state of the protein. In contrast, the high molecular weight fibrils and protofibrils collected in the pellet had the same level of toxicity as the non-centrifuged sample (Fig. 3). In previous studies showing the toxicity of A β oligomers, β -sheet-rich oligomers were isolated and concentrated from mature A β fibrils using size-exclusion chromatography or centrifugation through molecular weight cutoff membranes or density gradients, which led to the isolation of β -sheet-rich oligomers that were highly toxic to cells (Hartley et al. 1999; Hoshi et al. 2003; Chimon et al. 2007). Interestingly, it has been suggested that larger oligomers with a protofibrillar or spherical shape are more toxic than smaller oligomers (Ward et al. 2000; Hoshi et al. 2003; Chimon et al. 2007). Chimon et al. (2007) found that mature fibrils and β -sheet-rich oligomers with a molecular mass greater than 50 kDa were far more toxic to PC12 cells than oligomers smaller than 50 kDa. Similarly, Ward et al. (2000), using density-gradient centrifugation, showed that fractions containing protofibrils and mature fibrils were more toxic than those containing smaller oligomers. From our results using A β ₁₋₄₀, we conclude that the cytotoxic A β species are formed at later time points during the fibril-forming process (as the toxicity of the sample did not become significant until 48 h of incubation, see Fig. 7) and then, either the concentration of this cytotoxic A β species

Fig. 7 Time-dependent toxicity of $A\beta_{1-40}$. **a–d** Electron micrographs of $A\beta_{1-40}$ fibril formation after **a** 0 h, **b** 24 h, **c** 48 h, and **d** 72 h. Scale bar, 1 μ m. **e** $A\beta_{1-40}$ (10 μ M) was dissolved in phosphate buffer (50 mM, pH 7.4) and incubated at 37°C to induce fibril formation. Samples were taken over 0 to 72 h incubation and added to the cell culture media of the PC12 cells (0.5 μ M final concentration). The cells were incubated for 48 h followed by an MTT assay to assess cell survival. Values are presented as percentage of cell survival compared with control \pm SEM. * p <0.05, ** p <0.01 compared with 0 h incubation (n =4, one-way ANOVA, Dunnett's test)



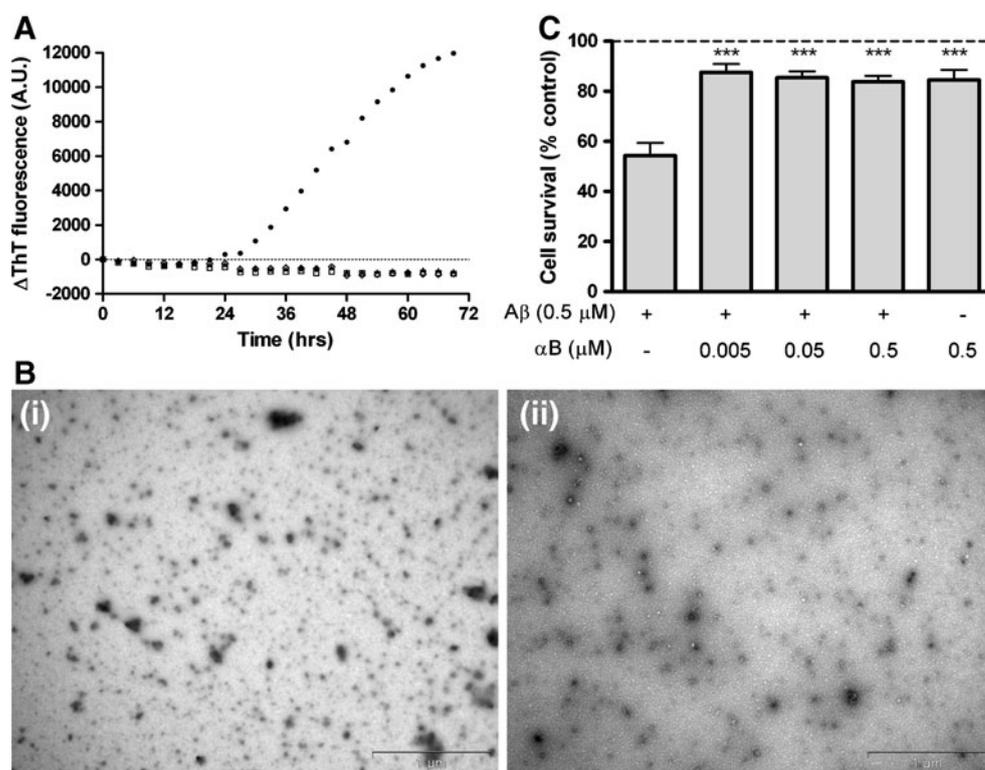
is maintained or more mature species, formed from it, have similar levels of toxicity. Similar techniques to those used in the studies on the cytotoxic $A\beta$ species could be employed to determine more precisely the relative toxicities of species formed during fibril formation by RCM κ -CN. Of note, with studies that apply particular forms of protein along its fibril-forming pathway, is that fibril formation still occurs in the incubation media used for the toxicity studies and, since the process of fibril formation is a dynamic one, changes in the relative amounts of each species during the incubation period need to be considered in interpretation of these data.

Previous studies in PC12 cells and other cell lines have shown that $A\beta$, α -synuclein, and the prion protein enhance cell toxicity through apoptotic mechanisms (Troy et al.

2000, 2001; O'Donovan et al. 2001; Tanaka et al. 2001; Onoue et al. 2002; Danzer et al. 2007). Furthermore, enhanced activity of apoptotic mediators such as caspases is observed in the brains of patients with AD and PD (Mattson 2006), which suggests that fibril-forming proteins cause cell death, in part, via apoptosis. Similarly, Hoechst staining showed that fibrillar RCM κ -CN enhanced chromatin condensation and fragmentation (Fig. 4), which indicates that RCM κ -CN causes PC12 cell death via apoptosis.

Previous research has shown that fibrillar aggregates of non-disease-related proteins are toxic, which suggests that amyloid fibrils share a common mechanism of toxicity (Bucciantini et al. 2002). Our study herein demonstrates that molecular chaperones are important in preventing

Fig. 8 Effect of αB-crystallin on Aβ₁₋₄₀ fibril formation and toxicity. **a** Aβ₁₋₄₀ (10 μM) was dissolved in phosphate buffer (50 mM, pH 7.4) and incubated for 72 h at 37°C in the absence (filled circle) or presence of αB-crystallin at 0.1 μM (square), 1 μM (filled triangle), and 10 μM (diamond); and fibril formation was measured by in situ ThT fluorescence. **b** Electron micrographs of Aβ₁₋₄₀ (10 μM) after 72 h in the presence of αB-crystallin at 0.1 μM (i) and 10 μM (ii). Scale bar, 1 μm. **c** Samples from the ThT assay were added to the PC12 cells so that the final concentration of Aβ₁₋₄₀ was 0.5 μM. The cells were incubated for 48 h followed by an MTT assay to assess cell survival. Values are presented as percentage of cell survival compared with control ± SEM. ****p* < 0.001 compared with 72 h Aβ₁₋₄₀ (*n* = 3, one-way ANOVA, Dunnett's test)



generic aspects of fibril formation and the toxicity associated with this process (Ecroyd and Carver 2009). Thus, αB-crystallin inhibited fibril formation by RCMκ-CN and Aβ, and this correlated with a reduction in the cell toxicity of these amyloid fibrils. In vivo, the upregulation of sHsps, which occurs in a variety of protein misfolding diseases, and their co-localization to amyloid deposits (Lowe et al. 1992; Shinohara et al. 1993; Renkawek et al. 1994; McLean et al. 2002; Pountney et al. 2005; Wilhelmus et al. 2006a, b), may represent an important protective effect to mitigate fibril formation and halt the progression of these diseases. Indeed, the recent observation for an intracellular phase of Aβ aggregation prior to extracellular fibril release (Friedrich et al. 2010) provides a plausible explanation for the collocation of αB-crystallin with Aβ in the extracellular plaques associated with AD.

One explanation for the contrasting results in the literature addressing the effect of αB-crystallin on the cell toxicity of Aβ (Stege et al. 1999; Wilhelmus et al. 2006a, b) is the methodology used to test the toxicity of Aβ. For both these studies, the native proteins were directly added to the cell media. As a result, it is unclear whether the effect of αB-crystallin on cell toxicity is directly related to its interaction with the fibril-forming protein or other components in the cell media. In previous studies showing the effect of Hsp20, another member of the sHsp family, on the toxicity of Aβ₁₋₄₀, the two proteins were pre-incubated before addition to the cell culture media (Lee et al. 2005, 2006) in a similar manner to that undertaken

herein. Pre-incubation has the advantage that the effect of αB-crystallin on the fibril-forming protein can be readily monitored by ThT and TEM and the species being added to the cell media (i.e., mature fibril, prefibrillar oligomer, amorphous aggregate, etc.) is well characterized. Lee et al. (2005, 2006) found that Hsp20 inhibited fibril formation by Aβ₁₋₄₀ and that the resultant Aβ₁₋₄₀ species were not toxic to PC12 and SH-SY5Y cells. However, other sHsps, Hsp17.7 and Hsp27, did not inhibit the toxicity of Aβ₁₋₄₀ despite preventing its fibril formation, and Aβ₁₋₄₀ species had a different morphology in the presence of Hsp17.7 and Hsp27 compared with Hsp20 (Lee et al. 2006) implying that Hsp17.7 and Hsp27 may not prevent the formation of smaller, toxic Aβ oligomers.

Consistent with our previous work (Ecroyd et al. 2007), αB-crystallin was not as efficacious in preventing RCMκ-CN fibril formation compared with other fibril-forming proteins. Thus, αB-crystallin caused a 67% inhibition of RCMκ-CN ThT fluorescence at a 2.0:1.0 mol/mol ratio. In comparison, αB-crystallin completely inhibited Aβ fibril formation at 0.01:1.0 mol/mol ratio, a potency similar to that reported by Raman et al. (2005). The marked difference in potency with these two target proteins may be due to the different kinetics of RCMκ-CN fibril formation compared with other amyloid proteins. Amyloid fibril aggregation occurs via a nucleation-dependent process where the formation of nuclei is the rate-limiting step. We have recently shown that RCMκ-CN aggregates via such a mechanism but the formation of nuclei is not the

rate-limiting step for fibril formation, as indicated by the lack of effect of seeding and absence of a protein concentration-dependent lag phase (Ecroyd et al. 2008). Once formed, the amyloidogenic precursor to RCM κ -CN fibril formation does not persist for long in solution and therefore, the ability of α B-crystallin to interact with and stabilize it is reduced. In contrast, fibril formation by A β_{1-40} has a long lag phase (approximately 20 h) providing significant opportunity for an interaction between α B-crystallin and partially folded intermediate states of A β_{1-40} . α B-Crystallin prefers to interact with slowly aggregating target proteins, whether they are doing so amorphously (Lindner et al. 2001) or in a fibrillar manner (Rekas et al. 2007). Thus, in diseases associated with fibril formation, conditions which increase the rate of fibril formation (e.g., mutations, stress, seeded fibrils) may exacerbate the disease due to a corresponding decrease in the effectiveness of the chaperone ability of sHsps to inhibit fibril formation.

The chaperone activity of α _S- and β -casein in preventing fibril formation by κ -casein (Thorn et al. 2005) also led to an inhibition of the RCM κ -CN-induced cell toxicity at mol/mol ratios of 0.8:1.0 and 1.6:1.0 (which are the approximate ratios found in milk for these proteins). Thus in vivo, the casein micelle in milk is important in stabilizing κ -casein to prevent its fibril formation and its potential toxic effects. This is of particular relevance to corpora amylacea, a disease characterized by amyloid-like deposits in bovine, rat, and canine mammary tissue (Reid 1972; Beems et al. 1978; Taniyama et al. 2000; Gruys 2004; Thorn et al. 2009). Bundles of fibrils have also been reported in the cytoplasm of cells that surround these calcified deposits (Nickerson 1987). Formation of fibrils associated with corpora amylacea is likely to be toxic to the surrounding cells which would be prevented by micelle formation of the casein proteins.

In summary, we have shown that RCM κ -CN is toxic to PC12 cells and that its toxicity can be attenuated by inhibiting its fibril formation utilizing molecular chaperones. RCM κ -CN is therefore a useful model for investigating the generic mechanism of fibril formation, the cytotoxicity associated with this process, as well as inhibitors of fibril formation that may be potential therapeutics for the treatment of fibril-associated diseases. We have used RCM κ -CN and A β as model fibril-forming proteins to directly demonstrate that the inhibition of fibril formation by the sHsp, α B-crystallin, leads to a decrease in cell toxicity. These results highlight the important role of sHsps in inhibiting the cell toxicity associated with fibril formation in protein misfolding diseases.

Acknowledgments We thank Dr. Lyn Waterhouse (Medical School, University of Adelaide) for assistance with the TEM and David Thorn for helpful conversations. This work was supported by a grant from the Australian Research Council to J.A.C. H.E. was supported by a National Health and Medical Research Council Peter Doherty Fellowship.

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