CELL BIOLOGY

Thioflavin S binds non-amyloid protein structures in lampbrush chromosomes of *Gallus gallus domesticus*

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Abstract

Proteins that normally function in amyloid form are found in bacteria, yeast, plants and vertebrates, including humans. In particular, amyloid fibrils and amyloid-like structures are described in the germ cells of various organisms. Recently we showed that in chicken oocytes there are some nuclear structures that are stained by the amyloid-specific dye thioflavin S. Here we demonstrate that thioflavin S binds giant terminal RNP aggregates in chicken lampbrush chromosomes. However, these structures are not stained with Congo red and conformation-dependent anti-amyloid antibodies. Thus, thioflavin S stains chromosome-associated proteins that do not have amyloid properties. These data indicate that thioflavin S must be used with caution when identifying new functional and pathological amyloids.

Keywords: amyloid, thioflavin S, Congo red, anti-amyloid antibodies, chicken, lampbrush chromosomes

Introduction

Amyloids are protein fibrils with a specific cross-β structure, which are usually associated with incurable diseases affecting the brain and other organs of humans and animals. At the same time, it was shown that a number of proteins normally function in amyloid form in a wide variety of organisms. Functional amyloids are found in bacteria, yeast, plants and vertebrates, including humans (Antonets et al., 2020; Sergeeva and Galkin, 2020). Amyloid fibrils possess unique mechanical strength, elasticity and resistance to a variety of agents (Mason and Shimanovich, 2018). Due to these properties, amyloid fibrils are a necessary and sometimes irreplaceable material for the formation of various functional extracellular and intracellular structures.

In particular, amyloid fibrils and amyloid-like structures are found in the germ cells of various organisms. Structures staining with amyloid-specific dyes have been detected in oocyte shells of insect, fish and mammalian oocytes. The *Antheraea polyphemus* (silk moth) oocyte shell binds Congo red and demonstrates yellow-green birefringence all over its surface (Iconomidou and Hamodrakas, 2008). The zona pellucida, a layer surrounding the plasma membrane of fish and mammalian oocytes, is formed by structural proteins which bind amyloid-specific dyes (Podrabsky, Carpenter and Hand, 2001; Egge, Muthusubramanian and Cornwall, 2015; Pimentel et al., 2019). The zona pellucida is not the only known structure with amyloidogenic properties essential for the fertilization process. Amyloid-like proteins are present in the spermatozoon acrosomal matrix, as well as outside the acrosomal membrane (Guyonnet, Egge and Cornwall, 2014). Moreover, amyloid-like structures have been identified inside *Xenopus* oocytes in Balbiani bodies, large non-membranous compartments that comprise RNA, mitochondria, and other organelles (Boke et al., 2016).

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We recently showed that in chicken oocytes there are some nuclear structures that are stained with thioflavin S dye, which binds various amyloid proteins (Siniukova et al., 2020). Here we identify these structures as giant loops associated with lampbrush chromosomes and analyze their amyloid properties. The detection of amyloid proteins in vivo is not an easy task. In vitro, the presence of a cross-β sheet amyloid structure can be proved using X-ray diffraction analysis or solid-state nuclear magnetic resonance, but these methods are suitable for the analysis of purified proteins and often cannot be used in the study of protein structures in vivo. In this regard, cytological dyes such as Congo red, thioflavin T and thioflavin S are used to prove the amyloid properties of the protein in vivo (Sergeeva and Galkin, 2020). Thioflavin T recognizes many but not all amyloid fibrils (Kabani and Melki, 2020), and also binds RNA and DNA quadruplex (Xu et al., 2016) as well as some non-amyloid proteins (De Ferrari et al., 2001; Carrotta et al., 2001). Its analogue, thioflavin S, does not stain nucleic acids, but its specificity for amyloid proteins is controversial. Specific staining with Congo red and yellow-green birefringence after such staining is considered as the golden standard for proving the amyloid properties of proteins in vivo (Benson et al., 2018, 2020), despite rare cases of false positive staining of non-amyloid aggregates with Congo red (Khurana et al., 2001; Bousset et al., 2004). In recent years, conformation-dependent amyloid-specific antibodies have also been used to detect amyloids directly in tissues through immunohistochemical reactions. These antibodies recognize the cross-β structure regardless of the amino acid sequence of proteins (Kayed et al., 2007). This is a promising approach, but the versatility of these antibodies for the detection of all amyloid proteins has not been shown. Taking into account all these data, to analyze the amyloid properties of chromosome-associated structures in chicken oocytes, we combined staining with cytological dyes with immunohistochemical analysis. The data obtained show that thioflavin S specifically binds non-amyloid protein structures in lampbrush chromosomes of *Gallus gallus domesticus*.

Materials and methods

Animals

Female adult (6-month-old) White Leghorn chickens were bought from commercial stock (Federal State Unitary Research Farm "Gene Pool" (Genofond), Pushkin, Leningrad Region, Russia).

Preparation of lampbrush chromosomes

Chicken lampbrush chromosomes were isolated manually from previtellogenic oocytes according to the standard technique (described in detail by Saifitdinova et al., 2017). After fixation in 2 % formaldehyde solution in PBS, preparations were kept in 70 % ethanol at +4 °C until their use.

Cytological staining

Thioflavin S staining: Lampbrush chromosomes were stained with 1 % thioflavin S (Sigma, USA) solution in 70 % ethanol in a humid chamber at room temperature for 5 min. Afterwards, staining preparations were washed three times for 5 min in 70 % ethanol, dehydrated in 96 % ethanol and air-dried. Preparations were mounted in antifade medium containing 1 % DABCO (1,4-diazabicyclo[2.2.2]octane) for further cytological analysis.

Congo red staining: Lampbrush chromosome preparations were successively rehydrated to 50 % ethanol and stained in 1 % Congo red (Reanal, Hungary) solution in ethanol in a humid chamber at room temperature for 10 min. Afterwards, staining preparations were washed three times for 5 min in 50 % ethanol, dehydrated in an ethanol series up to 96 % and air-dried. Preparations were mounted in antifade medium with 1 μg/mL DAPI (4′,6-diamidino-2-phenylindole).

Pepsin treatment: Rehydrated lampbrush chromosome preparations were washed for 5 min in 2xSSC, treated with 0.0002 % pepsin solution in 0.01 N HCl at +37 °C for 10 minutes and post-fixed with 1 % paraformaldehyde (in PBS, 50 mM MgCl2). Dehydrated preparations were stained with thioflavin S as described above.

Anti-amyloid fibrils (OC) antibody staining: To minimize nonspecific antibody binding, the rehydrated lampbrush chromosome preparations were incubated in 1 % blocking reagent (ThermoFisher Scientific, USA) in PBS for 1 h at room temperature. The primary conformation-specific antibody to amyloid fibrils (OC, ab2286, Sigma, USA) was used at a dilution of 1:500. The lampbrush chromosome spreads were incubated with primary antibodies overnight at +4 °C, and with the secondary anti-rabbit antibodies conjugated with Alexa Fluor 594 (ThermoFisher Scientific, USA) for 1 h at +37 °C. Both antibodies were diluted in PBS with 1 % blocking solution. The preparations were counterstained with DAPI and mounted in antifade solution.

Microscopy

Analysis of lampbrush chromosome preparations stained with amyloid-specific dyes thioflavin S and Congo red or with anti-amyloid fibrils (OC) antibodies was performed using a fluorescence Leica DM4000B microscope (Leica Microsystems GmBH, Germany) equipped with a black-and-white CCD camera and appropriate filter cubes (Leica Microsystems, Germany). Leica LAS AF software was used to acquire and process multicolour

images. Adobe Photoshop CS5 (Adobe Systems, USA) was used for figure assembling.

Proteomic screening and identification of proteins forming amyloid-like aggregates

Proteomic screening and identification of proteins forming amyloid-like aggregates in oocytes of *Gallus gallus domesticus* were performed using the PSIA–LC–MALDI approach described recently (Ryzhova et al., 2018). This approach is based on the isolation of proteins which form aggregates resistant to treatment with 1 % SDS at room temperature. Protein lysates were solubilized in Tris-buffered saline (TBS) (30 mM Tris-HCl, pH 7.4, 150 mM NaCl), supplemented with 10 mM PMSF and Complete Protease Inhibitor (Roche). The obtained lysates were fractionated by ultracentrifugation (151000 g, 2 h, 4 °C). Pellets containing protein aggregates were resuspended in TBS with 200 μg/ml RNase A (Thermo Fisher, USA), incubated for 15 min and treated with 1 % SDS for 8 hours at 18 °C. Then, detergent-resistant protein complexes were separated by ultracentrifugation at 151000 g (8 h, 18 °C) through 25 % sucrose-TBS cushion with 0.1 % SDS. Pellets were suspended in water, sedimentated again at 151000 g (2 h, 4 °C) and denatured. After trypsinolysis the peptide mixtures were loaded (1 μl) onto an Acclaim PepMap 300 HPLC reverse-phase column (150 mm, 75 μm, particle size 5 μm; Thermo Scientific, USA) and separated in an acetonitrile gradient (2–90 %) for 45 min using an UltiMate 3000 UHPLC RSLC nano high-performance nanoflow liquid chromatograph (Dionex, USA). Peptide fractions were collected every 10 s and loaded onto a 384-sample MTP AnchorChip 800/384 microtiter plate (Bruker Daltonics) using a Proteineer fc II spotter (Bruker Daltonics). Peptides were identified using the Ultraflextreme MALDI-TOF/TOF mass spectrometer (Bruker Daltonics, DE). MS-spectra for each peptide fraction were determined and analyzed using WARP-LC software.

Statistical data processing

Relative quantification of fluorescence intensity was determined by ImageJ 1.53a. Statistical analysis was performed using the Mann — Whitney test by Prism 9.1.2 (GraphPad Software, USA).

Results and discussion

We have recently shown that thioflavin S stains some chromosome-associated structures on cryosections of chicken previtellogenic oocytes (Siniukova et al., 2020). At this stage, avian oocytes contain transcriptionally active giant chromosomes, which are commonly called lampbrush chromosomes. Each chromosome has a characteristic chromomere-loop pattern represented by an array of condensed chromomeres with pairs of extended lateral loops, actively transcribed by RNA polymerase II (Callan, 1986). Besides, lampbrush chromosomes can possess "special loops" — nuclear domains enriched with RNA but not involved in on-going transcription. In chickens, one type is "terminal giant loops" (TGLs) (Chelysheva et al., 1990; Gaginskaya, Kulikova and Krasikova, 2009), later renamed GITERA, giant terminal RNP aggregates, accumulating poly-A RNA transcripts and RNA-binding proteins (Kulikova et al., 2015).

We made preparations of lampbrush chromosomes and stained them with thioflavin S. The results of this experiment are presented in Figure 1. Thioflavin S brightly colors GITERAs located at the termini of lampbrush chromosomes (Fig. 1, A).

To check that thioflavin S binds specifically to proteins, we pretreated the preparations of lampbrush chromosomes with pepsin. The data presented in Figure 1B show that after peptidase treatment, which breaks down protein molecules, thioflavin S does not stain lampbrush chromosomes. We also showed that the intensity of GITERAs staining with thioflavin S was significantly higher before pepsin treatment than after (Fig. 1, C). Thus, we can conclude that this dye stains the protein components of GITERAs.

The data obtained suggest, but do not prove, that the identified structures are of amyloid nature. To test this hypothesis, we stained lampbrush chromosome preparations with Congo red dye, as well as anti-amyloid conformation-dependent antibodies OC. In both cases, staining of chromosomes from four oocytes was analyzed. For detection of OC antibodies, the secondary antibodies conjugated with Alexa Fluor 594 were used. In these experiments, lampbrush chromosomes were counterstained with DAPI, since the fluorescence spectra of Congo red and secondary antibodies conjugated with Alexa Fluor 594 do not overlap with the fluorescence of DAPI. As was shown previously, DAPI shows much more intense fluorescence when binding DNA compared to RNA. In this regard, DAPI brightly stained the chromosome axis, but its fluorescence was almost not detected in areas of localization of RNA-enriched GITERAs (Fig. 2).

Surprisingly, neither Congo red nor amyloid-specific antibodies stained GITERAs (Fig. 2). The GITERA aggregates also showed no yellow-green birefringence after Congo red staining (not shown). Thus, these RNP complexes bind thioflavin S, but do not stain with Congo red. Thioflavin S is a highly sensitive, but insufficiently specific dye. Congo red is known to stain in vitro and in vivo all known amyloid proteins (Benson et al., 2020). In addition to staining in transmitted light, when Congo red binds to amyloids, specific fluorescence is observed in the red spectrum (Linke, 2000). Moreover, amyloids are characterized by a yellow-green birefringence after

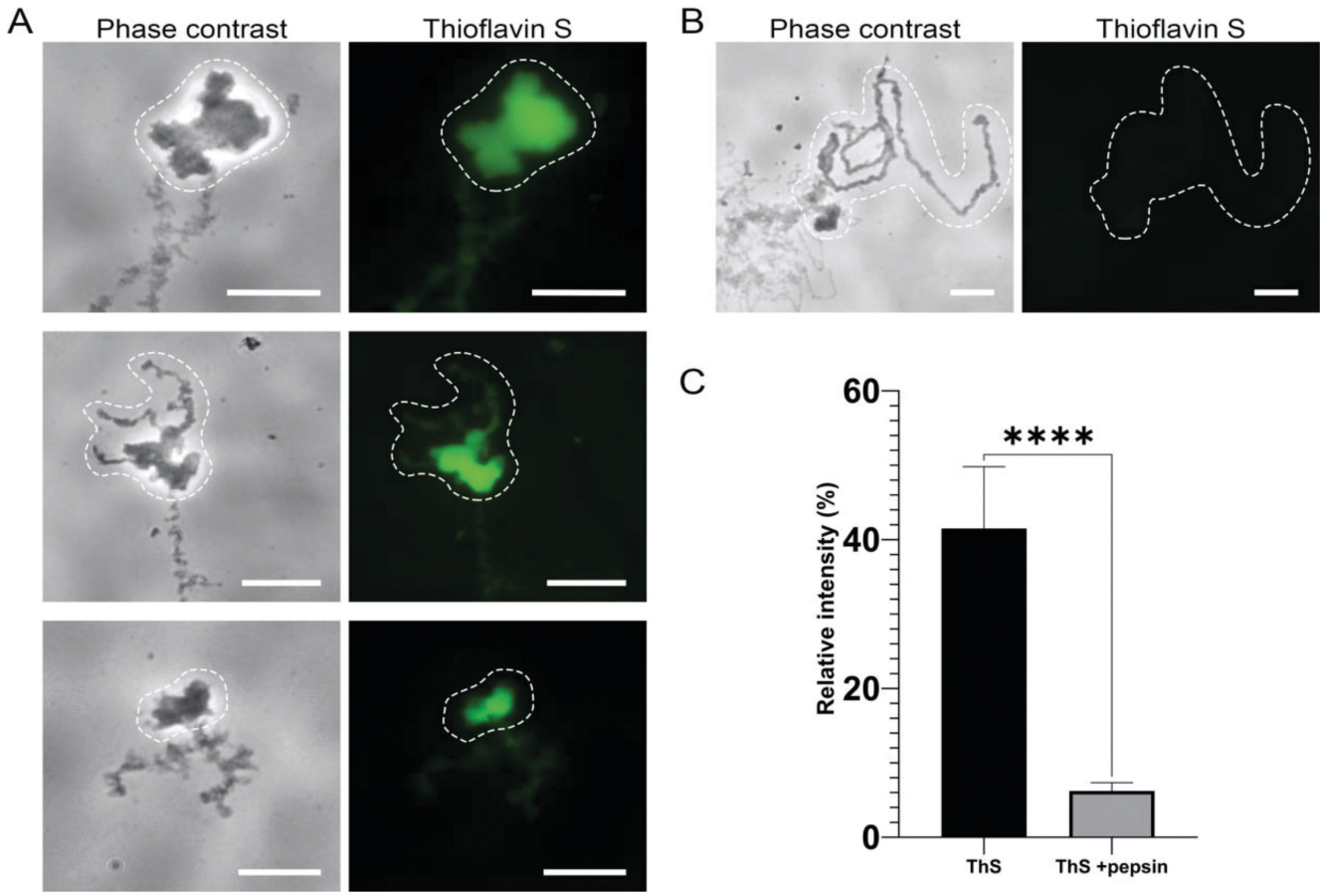

Fig. 1. Chicken lampbrush chromosomes stained with amyloid-specific dye thioflavin S. (A) Thioflavin S specifically binds GITERAs; (B) GITERAs do not bind thioflavin S after pepsin treatment. The GITERA localization area is outlined with a dotted line. Scale bars — 10 μm; (C) Comparative analysis of thioflavin S staining of GITERAs on chicken lampbrush chromosomes with and without pepsin treatment (black and gray columns, respectively). Relative fluorescence intensity of thioflavin S is represented as mean ± SEM. Quantification of relative fluorescence intensity was determined by ImageJ. Statistical analysis was performed using the Mann — Whitney test (* $p < 0.1$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$) by Prism.

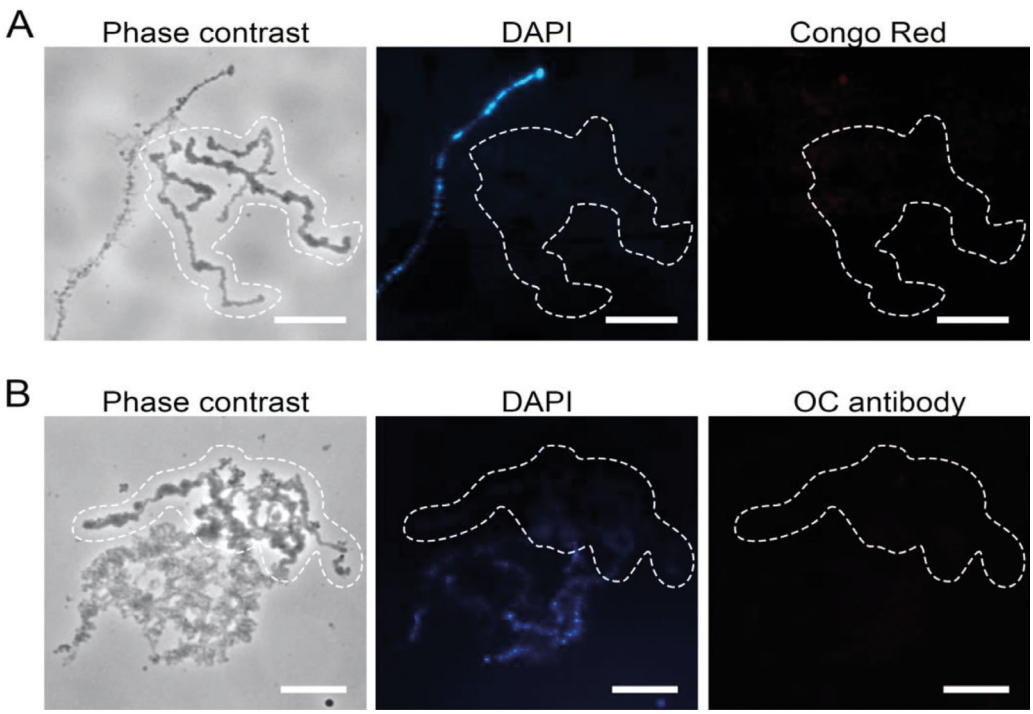

Fig. 2. Chicken lampbrush chromosomes after staining by amyloid-specific dye Congo red (A) and conformation-dependent Anti-Amyloid Fibrils OC Antibody (B). The GITERA localization area is outlined with a dotted line. Scale bars — 10 μm.

being stained with Congo red (Howie et al., 2008). In this regard, according to the official recommendations of the Alzheimer's Association, staining with Congo red is considered necessary proof of the amyloid nature of the protein. Thus, we can conclude that thioflavin S stains chromosome-associated proteins that do not have amyloid properties. The absence of binding of conformation-dependent anti-amyloid antibody (Fig. 2, B) supports this conclusion. A positive control for OC antibodies is shown in Supplementary Figure 1. These results can serve as an important warning for future studies on the search for and identification of amyloid structures using thioflavin S. This dye can obviously bind proteins that do not form amyloid fibrils.

So far, we can only speculate which proteins in GITERA are recognized by thioflavin S. The protein composition of these specific RNP aggregates is poorly understood. It is only known that GITERA are enriched in splicing factors SC35 and snRNPs (Krasikova et al., 2012; Kulikova et al., 2015). It is possible that thioflavin S binds a protein that forms ordered aggregates, which are structurally similar to amyloid fibrils. To get closer to solving this we performed proteomic screening of proteins that form amyloid-like detergent-resistant aggregates in chicken ovaries. Note that resistance to 1 % SDS at room temperature is a characteristic feature of amyloid fibrils and some other protein aggregates (Ryzhova et al., 2018). As a result of proteomic screening (3 independent experiments), we found that nuclear proteins cohesin PDS5, helicase CHD7, pre-mRNA processing factor PRPF3, and SMC5, involved in recombination repair, form detergent resistant aggregates (see Supplementary Tables 1–4). Considering that GITERA aggregates contain poly-A RNA and RNA-binding proteins, the most promising candidate is the PRPF3 protein, which interacts with poly-A RNA. This protein plays a role in pre-mRNA splicing, being a component of the U4 / U6-U5 tri-snRNP complex involved in spliceosome assembly (Liu et al., 2015). However, it should be borne in mind that so far this is only an assumption.

In conclusion, here we showed that thioflavin S binds non-amyloid protein structures in lampbrush chromosomes in chicken oocytes. These data indicate that thioflavin S must be used with caution when identifying new functional and pathological amyloids.

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