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Fibrinogen has chaperone-like activity

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Abstract

Partially or completely unfolded polypeptides are highly prone to aggregation due to nonspecific interactions between their exposed hydrophobic surfaces. Extracellular proteins are continuously subjected to stresses conditions, but the existence of extracellular chaperones remains largely unexplored. The results presented here demonstrate that one of the most abundant extracellular proteins, fibrinogen has chaperone-like activity. Fibrinogen can specifically bind to nonnative form of citrate synthase and inhibit its thermal aggregation and inactivation in an ATP-independent manner. Interestingly, fibrinogen maintains thermal-denatured luciferase in a refolding competent state allowing luciferase to be refolded in cooperation with rabbit reticulocyte lysate. Fibrinogen also inhibits fibril formation of yeast prion protein Sup35 (NM). Furthermore, fibrinogen rescues thermal-induced protein aggregation in the plasma of fibrinogen-deficient mice. Our studies demonstrate the chaperone-like activity of fibrinogen, which not only provides new insights into the extracellular chaperone protein system, but also suggests potential diagnostic and therapeutic approaches to fibrinogen-related pathological conditions.

Keywords

Fibrinogen; Chaperone; Extracellular; Aggregation; Misfolding; Fibril formation

Partially or completely unfolded polypeptides are highly prone to aggregation due to nonspecific interactions between their exposed hydrophobic surfaces [1,2]. Living systems have evolved elaborate mechanisms to prevent such interactions and help proteins fold correctly. Of particular significance in this regard are various chaperones present in abundance intracellularly [3,4]. Extracellular proteins are continuously subjected to stresses, such as free radicals, shear stress in blood, and elevated body temperature. However, the existence of extracellular chaperones that modulate the folding and stabilization of extracellular proteins remains largely unexplored [5]. A few extracellular proteins, such as clusterin, haptoglobin, and serum amyloid P component (SAP), have been reported to have certain chaperone-like activity in humans [6–8]. More extracellular chaperones still remain unknown.

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Human fibrinogen (FG) is a circulating 340 kDa glycoprotein, with a concentration of 2–4.5 mg/ml in the plasma [9]. FG is not only a vital part of the ‘‘common pathway” of the coagulation process [10], but also an acute-phase protein, the level of which increases under stress conditions [11]. FG binds to other extracellular matrix molecules and can act as a reservoir for growth factors, proteases and protease inhibitors. Elevated plasma FG is associated with age, atherosclerotic disease, acute myocardial infarction, and stroke [12,13]. However, the roles of FG in many of these physiological and pathological conditions are still not clear.

Here we show that FG has a chaperone-like activity. The chaperone-like property of FG was tested using model proteins for chaperone studies, such as citrate synthase (CS) [14] and luciferase [15,16]. Interestingly, FG can interact with partially denatured CS and protect it from thermal-induced aggregation and inactivation. Furthermore, FG can maintain thermal-denatured luciferase in a refolding competent state. FG also inhibits fibril formation of Sup35 (NM), the prion-determining domain of yeast prion protein Sup35 [17]. Moreover, FG rescues thermal-induced protein aggregation in the plasma of FG-deficient ($FG^{-/-}$) mice. Taken together, these studies indicate that FG has chaperone-like activity, which provides new insights into the extracellular chaperone protein system.

Materials and methods

Materials

Human plasma FG, fibronectin (FN), IgG, transferrin, pig heart CS, firefly luciferase, heat shock protein 90 (HSP90), and GroEL were purchased from Sigma (USA). Bovine serum albumin was obtained from Roche (CH). Purified rabbit polyclonal antibodies against CS were purchased from Nordic Immunology (NL). The generation of $FG^{-/-}$ mice has been described previously [18]. Polyclonal goat anti-rabbit immunoglobulin/HP was from DakoCytomation (DK). All other antibodies were from Protgen (CN).

Chaperone activity assays with CS

Light scattering and activity assays of CS were carried out as described [14]. To determine the aggregation kinetics, light scattering was measured in an FL-4500 fluorescence spectrophotometer (Hitachi, JP).

Co-immunoprecipitation (IP) was carried out as follows: CS with or without FG was incubated at 25 °C or 43 °C. The reactions were stopped after different time courses of incubation, and the samples were centrifuged. The complex of FG and CS in the supernatant was pulled down with polyclonal antibody against FG, which was subjected to Western blotting with polyclonal antibody against CS.

Luciferase reactivation experiments

Luciferase reactivation experiments were carried out as described [15]. Luciferase (1 μM) was incubated with 10 μM FG, BSA, human IgG, transferrin, or lysozyme, respectively, in the presence of 50 mM sodium phosphate, pH 7.5 (100 μl total) at 43 °C for 20 min, then cooled down to room temperature. The heated mixture was diluted by 40-folds into solutions containing 30 μl of rabbit reticulocyte lysate (RRL), 25 mM Hepes (pH 7.5), 5 mM $MgCl_2$, 10 mM KCl, 2 mM dithiothreitol (DTT), 2 mM ATP (50 μl total volume). In the parallel control studies, the heated mixture of luciferase and FG was diluted by 40-folds into Hepes buffer. Reactions were carried out at 30 °C. Aliquots were withdrawn at various time points, and luciferase activity was then measured using Centro LB 960 Luminometer from the Berthold Technologies GmbH & Co. KG. The activity of luciferase before incubation at 43 °C was assumed as 100% activity.

Thermal-induced protein aggregation in plasma of $FG^{-/-}$ mice was performed at 43 °C. After incubation for 48 h, the plasma from $FG^{-/-}$ mice and wild type mice was centrifuged at 20,000g for 15 min, and the precipitations were detected by SDS–PAGE. Bands in the gel were identified with liquid chromatography–tandem mass chromatography (LC–MS/MS).

Amyloid formation assays

The assay for amyloid formation was carried out as described [19]. Conversion to fibril was monitored by a change of thioflavin T based fluorescence intensity. Basically, concentrated stocks of Sup35 (NM), stored in 8 M urea, were diluted at least 50-fold into 5 mM KH_2PO_4, 150 mM NaCl, and pH 7.4. At indicated time, aliquots were measured in a FL-4500 fluorescence spectrophotometer (Hitachi), excited at 442 nm. Emission fluorescence at 485 nm was recorded. FG was added at distinct time points. Buffer only containing FG served as a control.

Results

Fibrinogen inhibits the thermal-induced aggregation and inactivation of citrate synthase

The ability to prevent aggregation of nonnative proteins is one of the fundamental biochemical properties for molecular chaperones [20]. To test whether FG has a chaperone-like activity, we employed citrate synthase (CS). CS is a model protein for chaperone studies, and the influence of the major classes of molecular chaperones on CS folding has been characterized [14].

The thermal-induced aggregation of 0.15 μM CS at 43°C was suppressed by FG in a dose-dependent manner, to an extent comparable to that found when clusterin, a previously reported extra-cellular chaperone protein [6], was present. On the other hand, high level (1.2 μM) of BSA showed no inhibition of CS aggregation (Fig. 1A). Other plasma proteins such as IgG, transferrin, and lysozyme were also tested, but no inhibitory effect was found (Fig. 1B). The suppression of CS aggregation by FG was not affected by ATP or its analog, ATPγS (Fig. 1B), and ATPase activity was not detected in FG (data not shown).

We also compared the ability of FG, clusterin, and HSP90 to protect the enzymatic activity of CS at 43 °C. Both 0.15 μM FG and 0.15 μM HSP90 dramatically attenuated the inactivation of CS, while 0.6 μM clusterin had significantly less protecting effect (Fig. 1C). To further confirm the specificity of the interaction between FG and partially denatured proteins, we tested whether FG selectively binds to the denatured form of proteins. Co-immuno-precipitation (IP) experiment was performed between FG and thermal-denatured CS. FG specifically binds to thermal-denatured CS at 43 °C but not native CS at 25 °C after co-incubation of these two proteins (Fig. 1D). These results indicate that FG can specifically interact with partially denatured CS and protect it from thermal-induced aggregation and inactivation.

Heat-denatured firefly luciferase or citrate synthase bound to fibrinogen are competent for refolding

We used firefly luciferase to further test whether a heat denatured protein bound to FG could still be competent for refolding. Luciferase was well studied as a model substrate for chaperone studies [15,16]. We found that FG inhibited thermal-induced aggregation of luciferase and formed complex with thermal-denatured luciferase (data not shown). After co-incubated with FG at 43 °C, the heated mixture of luciferase and FG was then added into rabbit reticulocyte lysate (RRL) to monitor the refolding process. RRL is rich in components needed for protein synthesis and folding, including molecular chaperones such as hsp70, hsp90, and TRiC [21]. Reactivation from the heated mixture of luciferase and FG reached nearly 12% of the original activity of luciferase before incubation (Fig. 2A). If luciferase was incubated alone without

FG, it irreversibly aggregated and no activity could be recovered by RRL (Fig. 2A). When luciferase was co-incubated with BSA, IgG, transferrin, or lysozyme, little reactivation of the heated mixture was obtained (Fig. 2B), thus the reactivation of luciferase was FG-dependent. However, FG itself alone was not enough to refold heat-denatured luciferase. When the heated mixture of luciferase and FG was added into Hepes buffer instead of RRL, little reactivation was obtained (Fig. 2B). In another thermal-induced inactivation test, CS was incubated without or with 0.6 μM FG, at 43 °C, until less than 5% of the original activity remained, and then temperature was reduced from 43°C to 25°C. Approximately 50% of the original activity of CS was recovered after 60 min, if initially incubated with FG. In contrast, less than 15% of the original activity of CS was regained, when this protein was initially incubated at 43 °C in the absence of FG (Fig. 2C). These results indicate that FG may serve as a reservoir, which maintains denatured proteins in a refolding competent form, so that the proteins can later be refolded either spontaneously or in conjunction with other chaperones. This property of FG is similar to some small heat shock proteins [15].

Fibrinogen inhibits fibril formation of Sup35 (NM)

Besides the protection to stressed proteins, FG was also found to have an inhibitory effect on the spontaneously protein misfolding and fibril formation. Yeast prion protein, Sup35 (NM), was employed for the study, because it can quickly form fibrils under physiological conditions and shares key features of amyloid fibrils of mammalian prions or amyloid proteins [17,22]. The fibril formation process of Sup35 (NM) was monitored by thioflavin T fluorescence, which was inhibited by FG in a dose-dependent manner (Fig. 3A). The inhibitory effect of FG on the fibril formation process was further observed by AFM (Fig. 3B and C). There were no differences between Sup35 (NM) alone and Sup35 (NM) co-incubated with FG at the beginning of incubation (Fig. 3B). Sup35 (NM) formed fibrils in the absence of FG (Fig. 3C, left panel) after 96 h, but mainly amorphous aggregates in the presence of FG (Fig. 3C, right panel). The result indicates that FG can modulate the aggregation process of fibril formation.

The inhibitory effect of FG on the fibril formation of yeast prion Sup35 (NM) once again demonstrates the chaperone-like activity of FG and is similar to the previous reported results of molecular chaperones [23,24].

FG prevents thermal-induced protein aggregation of plasma proteins

The general inhibition by FG of thermal-induced protein aggregation was further confirmed using plasma of $FG^{-/-}$ and wild type mice. After incubation at 43 °C for 48 h, a substantially larger amount of protein precipitate was found in the plasma of $FG^{-/-}$ mice, when compared to plasma of wild type mice (Fig. 4). This precipitation could also be rescued by adding exogenous FG into the plasma of $FG^{-/-}$ mice before incubation (data not shown). More than 12 proteins were identified in the precipitate by liquid chromatography–tandem mass chromatography (LC–MS/MS), which can be classified into four categories: coagulation proteins, extra-cellular matrix proteins, enzymes and other abundant plasma proteins (Table S1). Among these proteins, the binding of human plasminogen to fibrin and FG has already been studied [25]. Misfolding and amyloid fibrillogenesis of lysozyme variants associated with human amyloid disease has been characterized [26]. The protective effect of FG on the thermal-stressed precipitation of plasma proteins strongly suggests the biological relevance of FG.

Additional studies showed that purified FG significantly reduced the aggregation of chemical-denatured *Escherichia coli*-expressed nerve growth factor (NGF), PEX, and oxidatively damaged serum proteins (data not shown). Interactions of FG with such a wide spectrum of nonnative proteins portray its general chaperone-like feature.

Discussion

We show here that FG specifically interacts with and suppresses the aggregation of a wide spectrum of stressed proteins. To our knowledge, this is so far the first study to demonstrate the chaperone-like activity of FG. As a vital part of the coagulation system, human FG constitutively presents with a high concentration of 2–4.5 mg/ml in the plasma [9], and its level could increase by about 200% during the acute phase, such as inflammation and oxidative stress conditions [11]. Elevated expression of FG may be considered not only as a strategy for survival during unfavorable times but also as a potential mechanism of defense against misfolding events. Consistent with this point, misfolded proteins have been implicated for inflammatory events in atherosclerosis [27], and Alzheimer's disease [28]. Given the high concentration and important physical and pathological processes that FG gets involved, the chaperone-like activity of FG is of special significance to the large amount of extracellular proteins.

It is striking that FG can inhibit the formation of Sup35 (NM) fibril, which shares key features of amyloid fibrils of mammalian prions or amyloid proteins [22], indicating a potential role of FG on prion or other misfolding diseases. AFM study suggests that FG could modulate the aggregation process of Sup35 (NM) fibril to form amorphous aggregates (Fig. 3). Our result also indicates that FG more effectively inhibits Sup35 (NM) fibril formation at the early stage of fibril formation (data not shown). The property that FG can interact with the prefibrillar species during the fibril formation process not only supports the previous conception that chaperones can redirect the aggregation process toward the formation of amorphous deposits, thereby sequestering potentially toxic species from the bulk solution [23,24], but also provide strong therapeutic implication to the proposition that early prefibrillar soluble oligomers from proteins are inherently cytotoxic [29].

Recent observations revealed connections between the amyloid and hemostasis systems in humans [30–32]. It is particularly interesting that fibrin itself has high β-sheet content and fibrin-derived peptides can form amyloid fibers [32]. Several genetic variants in the region of the α-chain residues 522–554 in human FG were also reported to result in the formation of amyloid deposits [33–35]. FG was found to interact with non-digested PrP^{Sc} [36]. In FG-deficient mice, more advanced plaque formation in the artery was detected [37], supporting the contention that atherosclerosis may be a protein misfolding disease [27]. Interestingly, FG was also discovered to be associated with type II and cystic fibrosis-related diabetes [38,39], whereas type II diabetes is well known as being related to islet amyloid [40]. High levels of FG are also related to both Alzheimer's disease and vascular dementia [41]. It is plausible that the increased high level of FG may help to compensate the additional request of extracellular chaperone functions under such pathological conditions. Other possibilities cannot be excluded, such as the possibilities that FG molecules in Alzheimer's disease and vascular dementia may be mutated or contain abnormal post-translational modifications. Besides, human FG contains three potential integrin binding sites, two RGD sequences within the Aα chain and a non-RGD sequence in the γ chain. FG can interact not only with endothelia cells but also with immune cells such as monocytes, neutrophils, and B-lymphocytes, through both integrin and nonintegrin receptors [42]. Therefore, FG may also facilitate the cellular uptake and degradation of misfolded proteins to which it binds to.

Taken together, the novel chaperone-like activity of FG may not only provide new insights into the extracellular chaperone system but also have indications on FG-related physiological and pathological conditions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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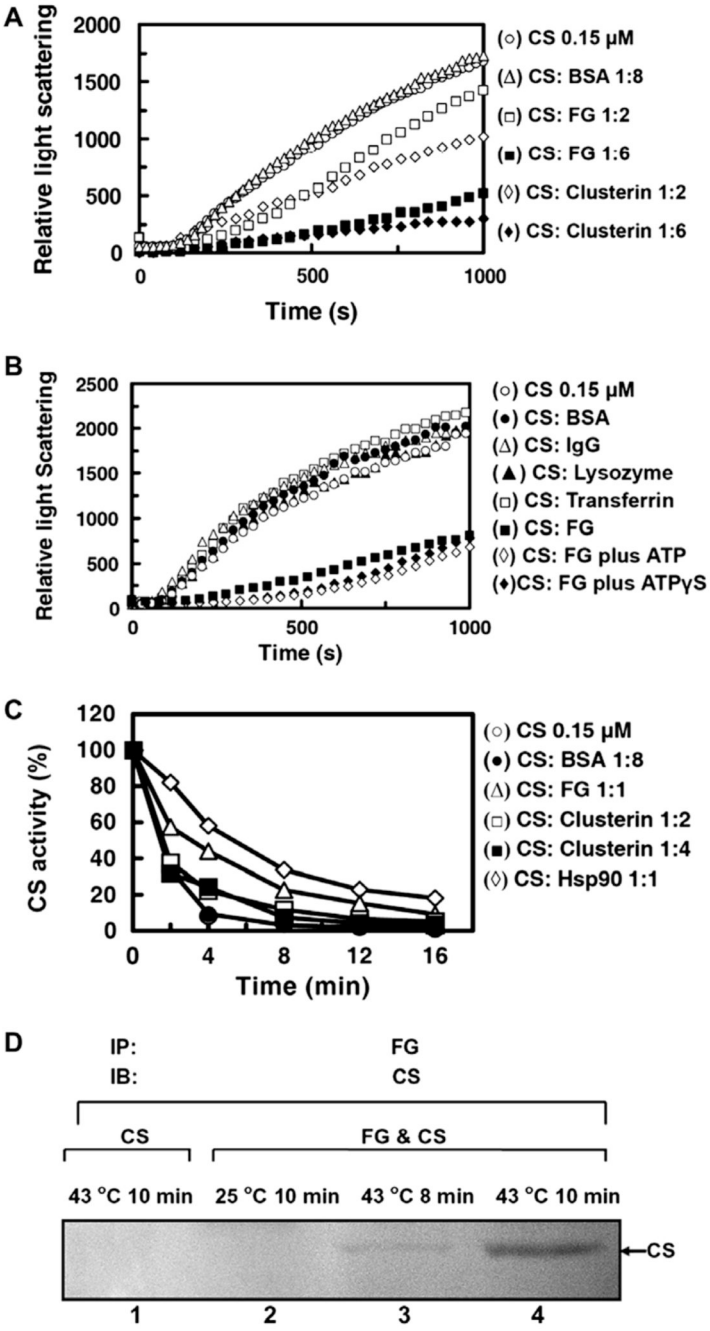

Fig. 1.

Chaperone activity of FG tested with citrate synthase (CS). (A) Thermal-induced CS aggregation. CS alone (○) or with 1.2 μM BSA (Δ), 0.3 μM FG (□), 0.3 μM clusterin (◊), 0.9 μM FG (■), 0.9 μM clusterin (♦). (B) The influence of ATP on thermal-induced CS aggregation. CS alone (○) or with 1.2 μM BSA (●), IgG (Δ), lysozyme (▲), transferrin (□), 0.9 μM FG (■), 0.9 μM FG plus 2 mM Mg ATP (◊), 0.9 μM FG plus 2 mM ATPγS (♦) at 43 °C. (C) Thermal-induced inactivation of CS at 43 °C. CS alone (○) or with 0.15 μM FG (Δ), 0.3 μM clusterin (□), 0.6 μM clusterin (■), 0.15 μM HSP90 (◊), 1.2 μM BSA (●). (D) Co-immunoprecipitation (IP) of FG and thermal-denatured CS. IP with antibody against FG; IB with antibody against CS.

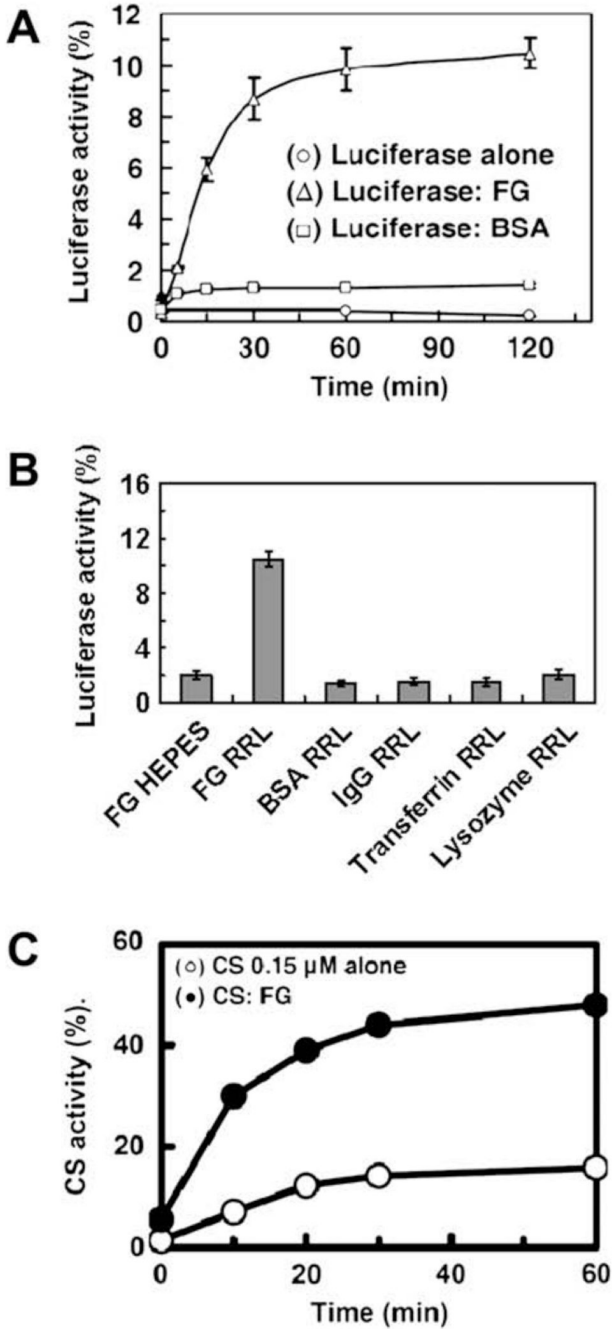

Fig. 2.

Heat-denatured firefly luciferase and CS bound to FG is competent for refolding. (A) Heated mixture of luciferase and FG added into rabbit reticulocyte lysate (RRL) (Δ), heated mixture of luciferase and BSA added into RRL (□), heated luciferase alone added into RRL (○). Error bars represent the standard error from duplicated measurements. (B) Heated mixture of luciferase and FG added into FG solutions, heated mixture of luciferase and FG added into RRL, heated mixture of luciferase and BSA, IgG, transferrin, lysozyme added into RRL. The height of the column represents the highest activity recovered. (C) Reactivation of CS. CS alone (○) or with 0.9 μM FG (●).

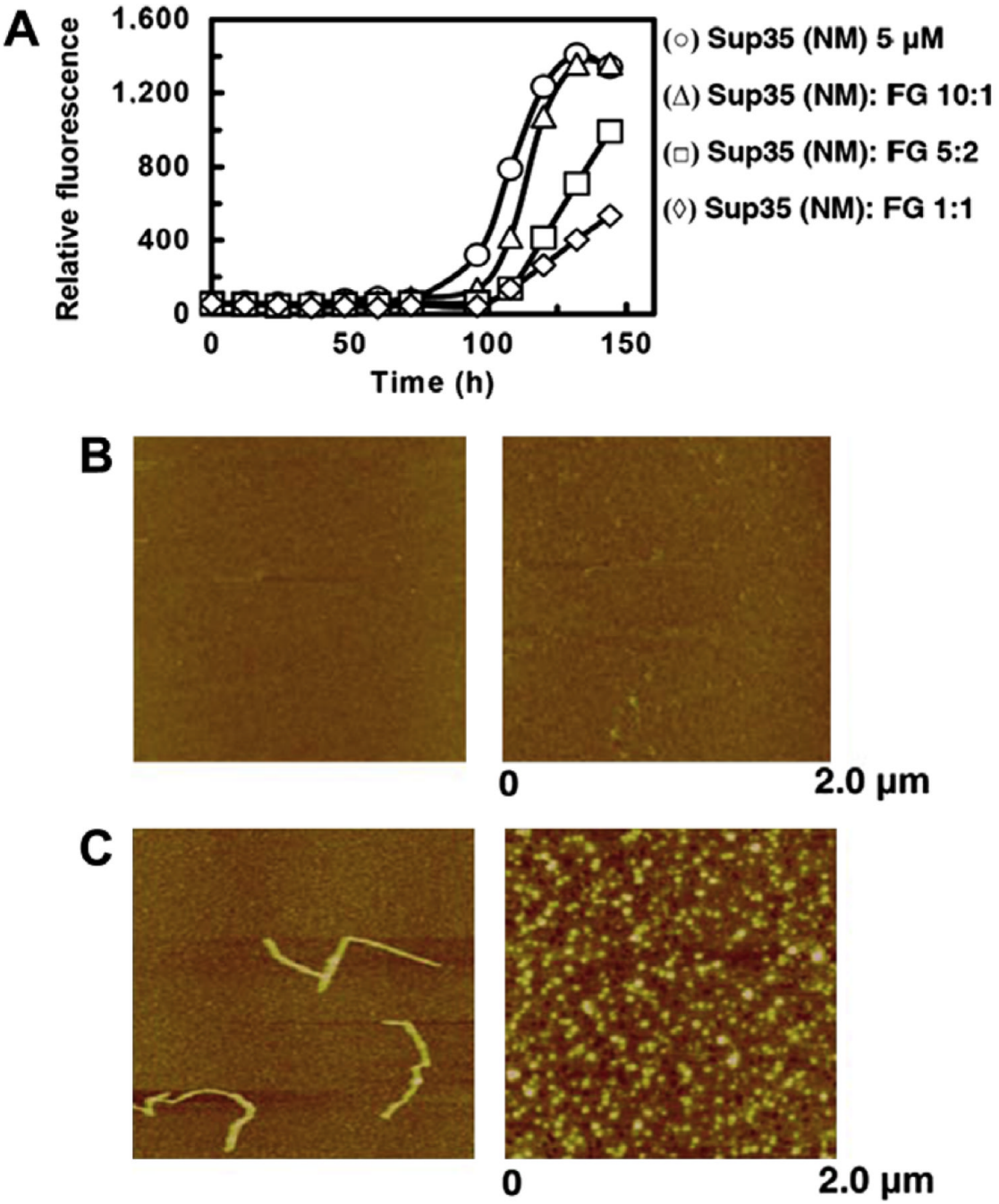

Fig. 3.

Inhibitory effect of FG on the process of fibril formation of yeast prion protein Sup35 (NM). (A) Fibril formation of Sup35 (NM) without FG (○) and with 0.5 μM (Δ), 2 μM (□), and 5 μM (◊) FG. (B) AFM profile of Sup35 (NM) without or with 5 μM FG after 24 h incubation. (C) AFM profile of Sup35 (NM) without or with 5 μM FG after 96 h incubation. In (B) and (C), left, without FG; right, with FG.

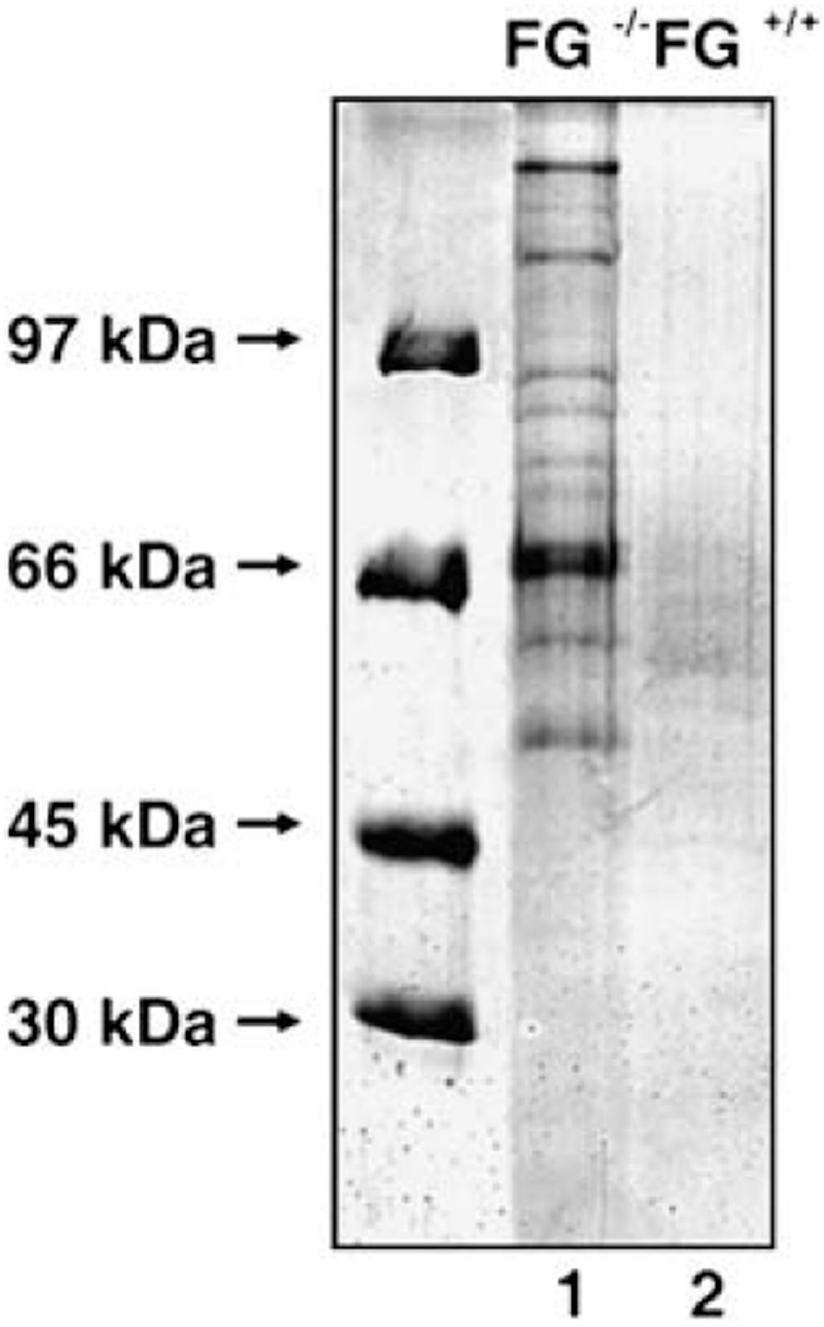

Fig. 4.
SDS–PAGE of precipitated proteins from plasma of $FG^{-/-}$ mice and wild type mice. Lane 1, precipitation in plasma from $FG^{-/-}$ mice; lane 2, precipitation in plasma from wild type mice.