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α_EC, the C-Terminal Extension of Fibrinogen, Has Chaperone-like Activity†

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ABSTRACT: Human fibrinogen is an important coagulation factor as well as an acute phase protein in the circulatory system. Fibrinogen-420 is distinguished from the conventional α chain of fibrinogen-340 by the presence of an additional 236-residue carboxyl terminus globular domain (α_EC). The α_EC domain of human fibrinogen-420 is a stable and early proteolytic cleavage product in the circulation. A genuine physiological function for α_EC has not yet been established. Our study aims to characterize the novel chaperone-like activity of α_EC. α_EC efficiently protects a series of model proteins from thermally induced aggregation. Furthermore, α_EC specifically recognizes the partially denatured form instead of the native form of citrate synthase (CS) and potentially protects it from thermally induced inactivation. The protective effect may result from formation of soluble complexes between α_EC and partially denatured CS as tested by size exclusion column and electron microscope. In addition, α_EC can keep the partially denatured luciferase in a folding competent state and help it refold in cooperation with rabbit reticulocyte lysate (RRL). Furthermore, α_EC can also form complexes with thermally stressed plasma proteins. Our findings reveal the novel function of α_EC as a chaperone-like protein, which not only provides new insights into the extracellular chaperone system but also has implications on the physiological and pathological relevance of fibrinogen.

Human fibrinogen is usually a circulating 340 kDa glycoprotein consisting of two identical disulfide-linked subunits, each of which is composed of three nonidentical polypeptides: α, *β*, and *γ* chains (*1*). Both *β* and *γ* chains have conserved C-terminal domains (designated as *β*C and *γ*C, respectively), which are distinct from the long, random coil at the carboxyl terminus of the conventional α chain (αC). Fibrinogen is not only a vital part in the "common pathway" of the coagulation process (*2*) but also an acute-phase protein, the level of which increases under stress (*3*). Elevated plasma fibrinogen is associated with aging, atherosclerotic disease, acute myocardial infarction, and stroke (*4*−*7*). Fibrinogen binds to other extracellular matrix molecules and acts as a reservoir for growth factors, proteases, and protease inhibitors (*8*). A 420 kDa isoform of fibrinogen (fibrinogen-420) has an alternative extended form of the α chain, which contains a unique 236-residue C-terminal extension termed α_EC[1] (*9*−*11*). The overall behavior of fibrinogen-420 was reported to have no significant difference compared with the predominant form of fibrinogen, named fibrinogen-340, in the clotting process and proteolytic susceptibility (*12*). (Fibrinogen-340 is named for the conventional 340-kDa $(\alpha\beta\gamma)2$ form, and fibrinogen-420 is named for the $(\alpha_E\beta\gamma)2$ form with its predicted mass of 420 kDa.) However, the gene of the fibrinogen-420 isoform, especially the α_EC domain, is highly conserved among vertebrates (*9*,*13*,*14*). The distinctive role of fibrinogen-420 compared with fibrinogen-340 has not been elucidated yet.

The α_EC shares approximately 40% amino acid identity with *β*C and *γ*C domains, and their three-dimensional structures are quite similar, as shown by X-ray crystallography (*15*−*17*). The α_EC domain contains a calcium-binding site but lacks the polymerization pocket in *β*C and *γ*C domains, so that α_EC does not participate in cross-linking during the coagulation process, indicating a different role of α_EC (*18*). The α_EC domain of human fibrinogen-420 was reported as a stable and early plasmin cleavage product, which could be detected in the plasma collected from patients with myocardial infarction shortly after the initiation of thrombolytic therapy (*12*). This finding implicates an independent function of α_EC in the

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[1]Abbreviations: α_EC, C-terminal domain of α chain; CS, citrate synthase; RRL, rabbit reticulocyte lysate; sHSPs, small heat shock proteins; HMW, high molecular weight; DTT, dithiothreitol; ATP, adenosine triphosphate; SEC, size exclusion column; Hsp90, heat shock protein 90; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MDH, malate dehydrogenase; Co-IP, coimmunoprecipitation.

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circulation. Recent experiments with recombinant α_EC expressed in *Escherichia coli* suggest that this domain is capable of supporting integrin-mediated cell adhesion (*19*). α_EC was also identified to be able to bind with leukocyte integrins $\alpha_M\beta_2$ and $\alpha_X\beta_2$ to mediate leukocyte adhesion and migration (*20*). Nevertheless, the physiological functions of α_EC are still poorly known.

Extracellular proteins are continuously subjected to stress conditions such as free radicals, shear stress in blood, and elevated body temperature, which could make extracellular proteins become misfolded and aggregated. However, the existence of extracellular chaperones that modulate the folding and stabilization of extracellular proteins remains largely unexplored (*21*). A few extracellular proteins such as clusterin, haptoglobin, α2-macroglobulin, and β-casein have been reported to have a chaperone-like activity (*22*−*26*). In our previous studies, we discovered that fibrinogen exhibited a chaperone-like activity, which was suggested to protect plasma proteins under stress conditions (*27*). Here we show that the 27-kDa α_EC domain of fibrinogen-420 can function independently as a chaperone-like protein similar to that of small heat shock proteins (sHSPs). α_EC can bind to the hydrophobic regions of partially denatured, stressed proteins and thereby inhibits their stress-induced aggregation and inactivation. The protective effect may result from the formation of soluble high molecular weight (HMW) complexes between α_EC and partially denatured proteins as tested with citrate synthase (CS). α_EC can keep the partially denatured proteins in a folding competent state in the complex and help them to refold in cooperation with other chaperone proteins under appropriate conditions. Taken together, our findings demonstrate that α_EC has a novel chaperone-like activity. The novel function of α_EC not only provides new insights into the extracellular chaperone system but also implicates potential diagnostic and therapeutic approaches to fibrinogen-related pathological conditions.

EXPERIMENTAL PROCEDURES

Materials. Human fibrinogen-420 and fibrinogen-340 were purified as described (*12*,*28*). α_EC was expressed and purified as described (*19*). Pig heart CS, firefly luciferase, Hsp27, Hsp90, human β-casein, and GroEL/S were purchased from Sigma. Bovine serum albumin and protein A−agarose were obtained from Roche. Dithiothreitol (DTT) was obtained from Sigma. Purified rabbit polyclonal antibodies against CS were purchased from Nordic Immunology. All other antibodies were from Protgen.

Aggregation Assay of CS, GAPDH, MDH, and Insulin. Light scattering was used to examine the influence of fibrinogen-420 and α_EC as described (*29*). To determine the aggregation kinetics, light scattering was measured with an FL-4500 fluorescence spectrophotometer (Hitachi). Both the excitation and emission wavelengths were set to 500 nm with a spectral bandwidth of 2.5 nm. Fibrinogen-420 and α_EC do not contribute to the light scattering signal at 500 nm, where only larger aggregates are detectable.

The DTT-induced aggregation of insulin was performed as described (*25*,*26*).The aggregation process was monitored at 360 nm with a UV-8500 spectrophotometer (Shanghai Techcomp). The reduction of insulin was initiated by the addition of 20 mM DTT into reaction buffer (50 mM Na_3PO_4, pH 7.4).

Activity Assay of CS. Thermal inactivation of CS and reactivation of chemically denatured CS were performed as described previously (*29*). For thermal inactivation, CS was incubated with or without α_EC at 43 °C in 40 mM HEPES−KOH, pH 7.5. For reactivation of chemically denatured CS, CS was first denatured in 6 M guanidinium chloride (in 50 mM Tris-HCl, pH 8.0) and 20 mM dithiothreitol (DTT) and then renatured by diluted 1:100 into renaturation buffer (40 mM HEPES−KOH, pH 7.5, with or without α_EC) with a final concentration of 0.15 μM CS monomer. To determine CS activity, aliquots were taken at the time points indicated, and the activity was measured (*29*). The activity measurements were carried out at 25 °C in 50 mM Tris-HCl and 2 mM EDTA, pH 8.0.

Formaldehyde Cross-Linking. Formaldehyde cross-linking was performed as described with modifications (*30*). Briefly, human plasma was diluted with PBS (1:1 v/v). Then aliquots of 400 μL of diluted plasma were added with 2 μM α_EC and incubated at 37, 43, and 50 °C for 30 min, respectively. The control sample did not have α_EC added but was incubated at 37 °C for 30 min. After incubation, the samples were centrifuged for 10 min at 12000 rpm at 4 °C. Cross-linking was performed by adding 1% formaldehyde into the supernatant and incubating at 30 °C for 10 min. To stop the cross-linking reaction, 1.25 M glycine was added to a final concentration of 125 mM for 5 min at 30 °C. The samples were loaded onto SDS−PAGE to test the existence of HMW protein complexes. The α_EC monomer in the plasma was detected with Western blotting.

Coimmunoprecipitation. Co-IP between α_EC and CS was carried out as follows: CS (0.3 μM subunit) and α_EC (0.3 μM) were incubated at 25 or 43 °C in 40 mM HEPES. The reactions were stopped after different time courses of incubation, and the samples were centrifuged at 4 °C. The complexes of CS and α_EC in the supernatant were pulled down with polyclonal antibody against CS or α_EC, respectively, at 4 °C, which were subjected to Western blotting with polyclonal antibody against α_EC or CS accordingly.

For the Co-IP in the plasma, human plasma was diluted by four times with PBS and incubated with protein A−agarose for 2 h at 4 °C to deplete IgG. Then 1 μM α_EC was added to the aliquots of 400 μL of diluted plasma and incubated at 37 or 50 °C for 30 min, respectively. The control sample which did not contain α_EC was incubated at 37 °C. After incubation, the samples were centrifuged for 10 min at 12000 rpm at 4 °C. The complexes formed between α_EC and substrate proteins in the supernatant were pulled down by polyclonal antibody against α_EC and checked by SDS−PAGE. The Co-IP process was performed as described in the manual for protein A−agarose (Roche). Bands in the gel were identified with liquid chromatography−tandem mass chromatography (LC-MS/MS).

Luciferase Activity Assay. Luciferase reactivation experiments were carried out as described (*31*). Luciferase (1 μM) was incubated with or without 10 μM α_EC in the

presence of 50 mM sodium phosphate, pH 7.5 (100 μL total), at 43 °C for 20 min and then cooled down to room temperature. The heated mixture was diluted by 40-fold into solutions containing 30 μL of RRL, 25 mM HEPES (pH 7.5), 5 mM $MgCl_2$, 10 mM KCl, and 2 mM DTT with or without 2 mM ATP (50 μL total volume). In the parallel control studies, luciferase was heated alone in HEPES and diluted by 40-fold into RRL with ATP. Reactions were carried out at 30 °C. Aliquots were withdrawn at various time points and diluted 500-fold into 25 mM HEPES buffer (pH 7.5). Luciferase activity was then measured using a Centro LB 960 luminometer from Berthold Technologies GmbH & Co. KG. The activity of luciferase before incubation at 43 °C was assumed to be 100%.

Analysis of α_EC−CS Complexes. CS (1 μM subunit) and α_EC (1 μM) were incubated at 25 or 43 °C in 40 mM HEPES. The reactions were stopped after 10 min of incubation, and the samples were centrifuged at 4 °C. Supernatants were loaded onto a size exclusion column (SEC) using Superdex 200 at 25 °C. For the analysis of component proteins within HMW complexes, the corresponding peak fractions were pooled, concentrated, and analyzed with SDS−PAGE. The molar ratio of α_EC versus CS subunit in the complexes was calculated with software (TotalLab v2.01).

RESULTS

Fibrinogen-420 Has Chaperone-like Activity. The schematic structures of fibrinogen-420 and fibrinogen-340 are shown in Figure 1A. We purified fibrinogen-420 and fibrinogen-340 from human umbilical cord blood as described previously (*12*,*28*). As was shown in Figure 1B, the three major bands in lane 1 correspond to the conventional α, β, and γ chains of fibrinogen-340, while the band of more than 97 kDa in lane 2 represents the α_E chain of fibrinogen-420. There were also partially proteolyzed forms in lane 2 at the position of the conventional α chain in lane 1. All of these results were consistent with the previous report (*12*).

We then compared the two isoforms of fibrinogen using citrate synthase (CS) as a model protein. Fibrinogen-420 displayed a predominant inhibitory effect on the thermally induced aggregation of CS. The thermally induced aggregation of CS at 43 °C was suppressed by fibrinogen-420 in a dose-dependent manner. Surprisingly, fibrinogen-340 showed little inhibitory effect on CS aggregation and behaved similarly to BSA (Figure 1C).

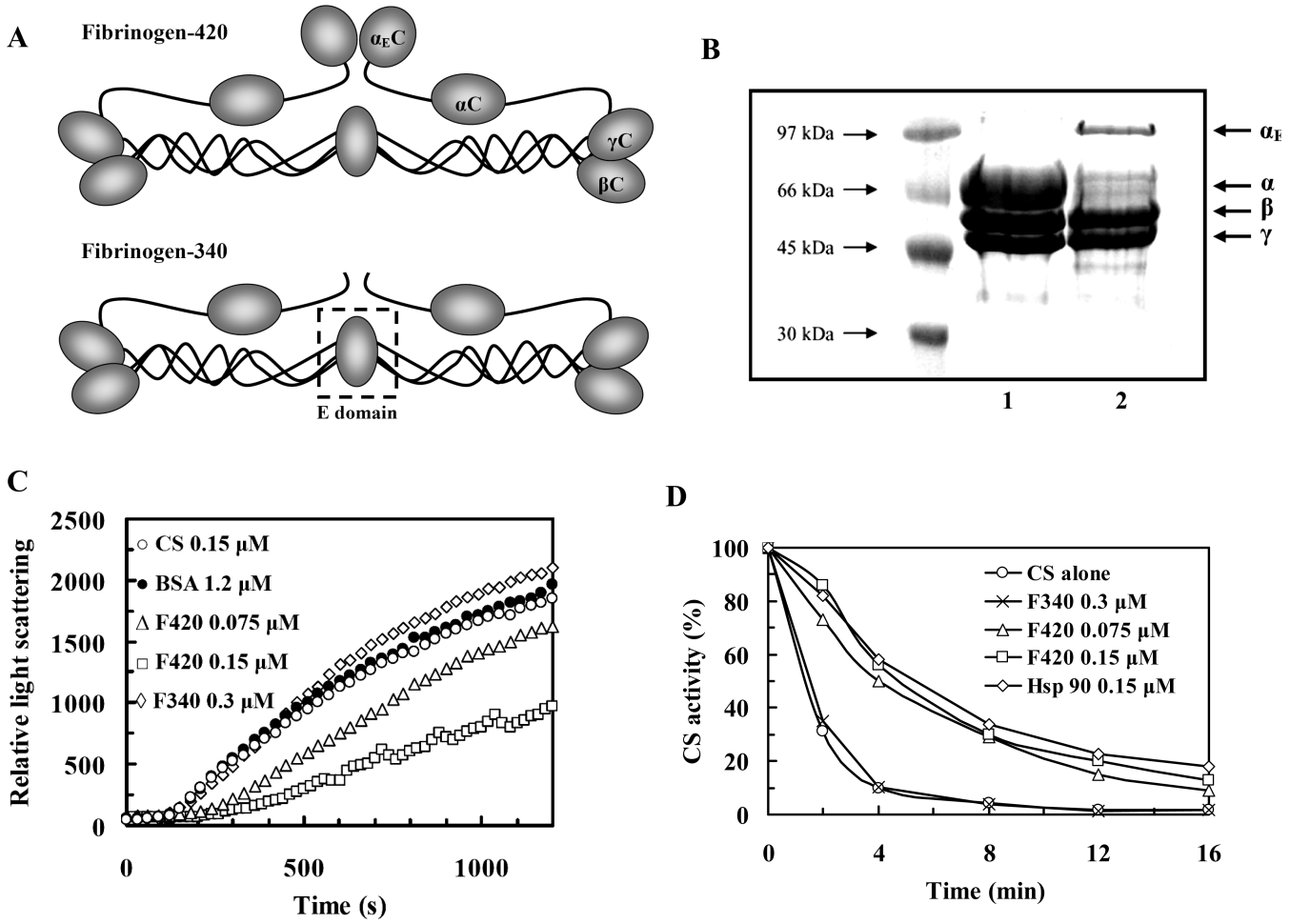

FIGURE 1: Fibrinogen-420 has chaperone-like activity. (A) Schematic figure of fibrinogen-420 and fibrinogen-340. The C-terminal domains of the α, β, and γ chain are as indicated. (B) SDS−PAGE of purified fibrinogen-420 and fibrinogen-340: lane 1, fibrinogen-340; lane 2, fibrinogen-420. (C) The kinetics of thermally induced CS aggregation was determined by light scattering: 0.15 μM CS incubated alone (○) or with 1.2 μM BSA (●), 0.075 μM fibrinogen-420 (△), 0.15 μM fibrinogen-420 (□), and 0.3 μM fibrinogen-340 (◇), respectively. (D) Thermally induced inactivation of 0.15 μM CS alone (○) or with 0.3 μM fibrinogen-340 (×), 0.075 μM fibrinogen-420 (△), 0.15 μM fibrinogen-420 (□), and 0.15 μM Hsp90 (◇), respectively. In panels C and D, the results are representative of two or more individual experiments.

Furthermore, we compared the ability of fibrinogen-420, fibrinogen-340, and heat shock protein 90 (Hsp90) to protect the enzymatic activity of CS at 43 °C. Fibrinogen-420 dramatically attenuated the inactivation of 0.15 μM CS at concentrations of 0.075 and 0.15 μM with an effect comparable to using 0.15 μM Hsp90, while fibrinogen-340 exhibited no protective effect (Figure 1D). These results indicate that the fibrinogen-420 isoform is predominantly responsible for the chaperone-like activity of fibrinogen.

α_EC Can Protect Different Proteins from Stress-Induced Aggregation. The major difference between fibrinogen-420 and fibrinogen-340 is that fibrinogen-420 has the extended C-terminal domain of α chain (α_EC) (*10*). Thus it is highly possible that the α_EC domain plays an important role in mediating the chaperone-like activity of fibrinogen-420. To verify this hypothesis, we prepared the recombinant α_EC domain as described (*19*) and tested the chaperone-like activity of the α_EC domain. As shown in Figure 2A, 0.05 μM α_EC dramatically inhibited the thermally induced aggregation of 0.15 μM CS with efficiency comparable to 0.15 μM Hsp27. In addition, 0.15 μM α_EC completely suppressed the aggregation of CS as 0.15 μM Hsp90 did. On the other hand, a high concentration (1.2 μM) of BSA showed no inhibitory effect. The inhibitory effect of α_EC was more potent than fibrinogen-420 containing equal molar concentration of α_EC (Figures 1C and 2A), probably because free α_EC binds more easily to substrates than being fixed to the end of fibrinogen-420.

Moreover, we observed that the chaperone-like activity of α_EC was not restricted to CS. α_EC could completely suppress the thermally induced aggregation of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), malate dehydrogenase (MDH) (Figure 2B,C), and luciferase (data not shown). α_EC could also inhibit the aggregation of insulin induced by reducing agents like DTT (Figure 2D). Compared with β-casein, the previously identified extracellular chaperone-like protein (*25*,*26*), even lower amounts of α_EC can significantly inhibit the aggregation process. These results strongly indicate that α_EC has a chaperone-like activity, which can protect different proteins from stresses such as thermally and chemically induced aggregation.

The Physical Interaction between α_EC and Thermally Denatured CS. The direct interaction between thermally denatured CS and α_EC was confirmed by coimmunoprecipitation (Co-IP) (Figure 3A). CS and α_EC were incubated together at 25 or 43 °C. At different time points, the supernatant was tested for the existence of CS−α_EC complexes. After coincubation at 43 °C, CS could be pulled down by the antibody against α_EC and vice versa, while no such binding was observed at 25 °C. The result indicates that α_EC specifically recognizes and binds to the thermally denatured form of CS instead of its native form.

The CS−α_EC complexes were further analyzed by SEC (Figure 3B). At the molar ratio of 1:1 (α_EC:CS subunit), incubation of the two proteins at 25 °C resulted in two clearly separated peaks referring to CS and α_EC. A new peak mainly eluted in the void volume of the column

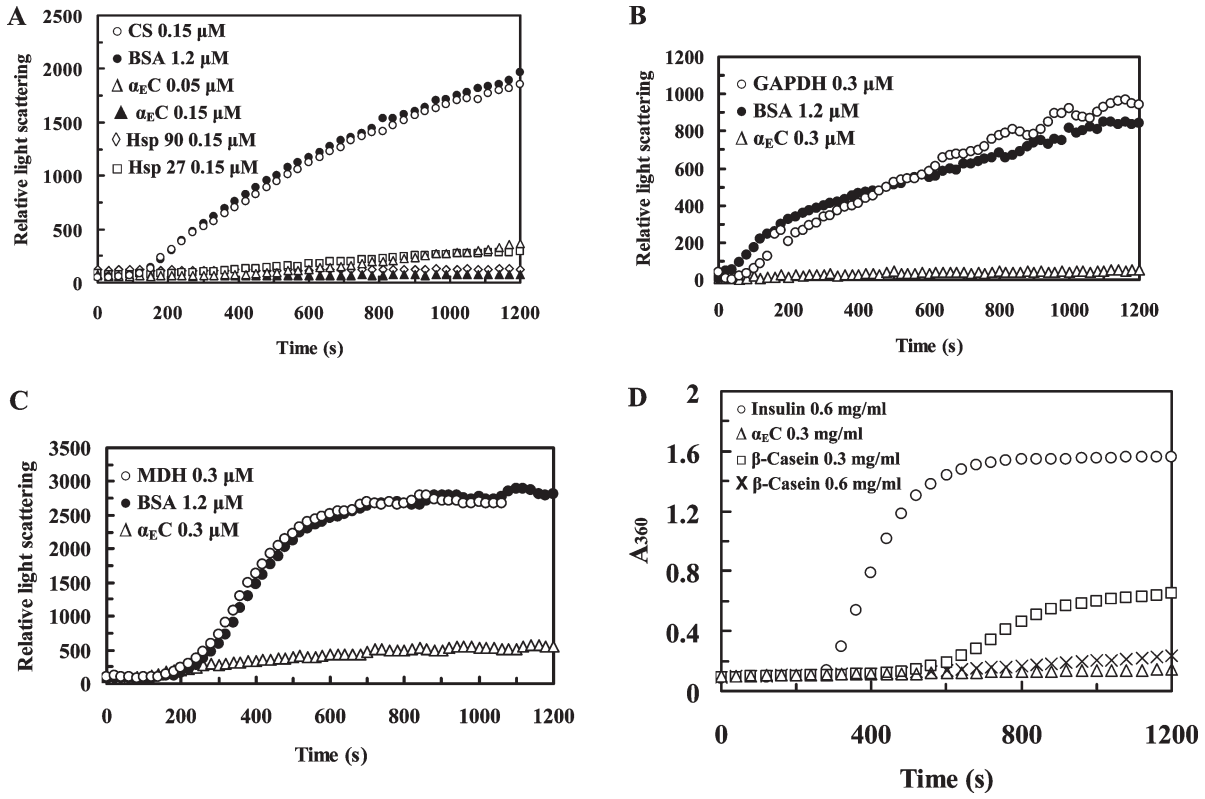

FIGURE 2: Thermally induced aggregation of CS, GAPDH, MDH, and insulin. (A) The kinetics of thermally induced CS aggregation was determined by light scattering. CS was incubated alone (○) or with 1.2 μM BSA (●), 0.05 μM α_EC (△), 0.15 μM α_EC (▲), 0.15 μM Hsp90 (◊), and 0.15 μM Hsp27 (□), respectively. (B) GAPDH alone (○) or with 1.2 μM BSA (●) and 0.3 μM α_EC (△), respectively. (C) MDH alone (○) or with 1.2 μM BSA (●) and 0.3 μM α_EC (△), respectively. (D) 0.6 mg/mL insulin (50 μM) alone (○), 0.3 mg/mL (10 μM) β-casein (□), 0.6 mg/mL (20 μM) β-casein (×), and 0.3 mg/mL (11 μM) α_EC (△). The results are representative of two or more individual experiments.

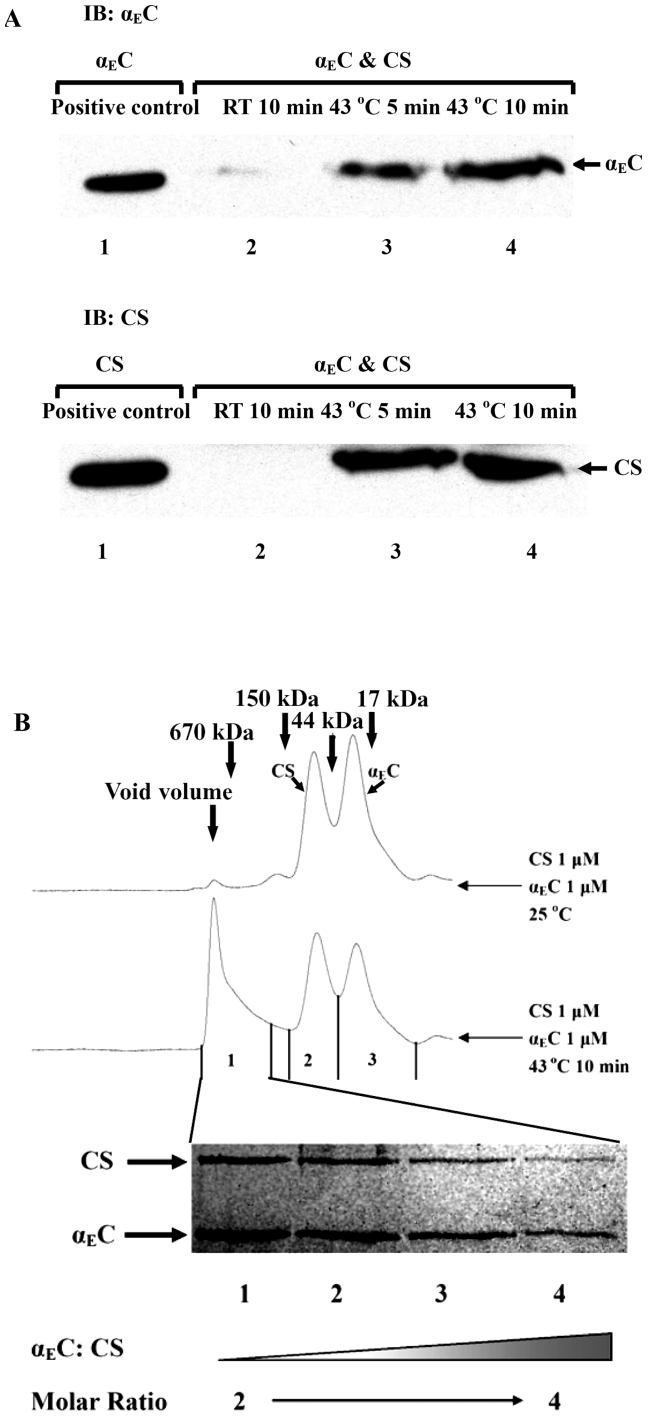

FIGURE 3: Detection of interaction between α_EC and CS by Co-IP and SEC. (A) Co-IP of α_EC and thermally denatured CS. Upper panel: IP with antibody against CS; IB with antibody against α_EC. Lower panel: IP with antibody against α_EC; IB with antibody against CS. (B) α_EC (1 μM) and CS (1 μM) were first incubated together at 25 or 43 °C, respectively, and then applied to the SEC as described in Experimental Procedures. For the SDS−PAGE analysis of isolated complexes of α_EC and CS, lanes 1−4, aliquot fractions were collected from peak 1 at different retention times from early to late.

appeared if both of the proteins were previously incubated together at 43 °C (Figure 3B). SDS−PAGE analysis revealed that the new peak (peak 1) contained both α_EC and CS (Figure 3B), indicating that binding of denatured CS to α_EC at elevated temperature resulted in the formation of HMW complexes. As was analyzed with software (TotalLab v2.01), the calculated molar ratio of α_EC versus CS subunit in the complexes ranged from 2 to 4 (Figure 3B). The lowest ratio appeared mainly in the void volume of the column. The lower the ratio, the higher the molecular weights of the complexes, which indicates that the binding capacity of α_EC saturates at a ratio of 2. A similar result was also reported for Hsp18.1, a small heat shock protein (*31*).

α_EC Inhibits Aggregation of Chemically Denatured CS. Influence of molecular chaperones on aggregation of chemically denatured CS was studied by Buchner (*29*). Interestingly, we found that α_EC can inhibit the aggregation of chemically denatured CS in a dose- and temperature-dependent manner (Figure 4). At 25 °C, guanidinium-denatured CS aggregated within 400 s upon dilution with HEPES buffer to a final concentration of 0.15 μM. While BSA had no effect on the aggregation process (Figure 4A), 0.6 μM α_EC could obviously inhibit the aggregation of CS. The inhibitory effect was more dramatic when using 0.9 μM α_EC. Most interestingly, when the aggregation assay was performed at 37 °C, the chaperone-like activity of α_EC dramatically increased (Figure 4B). Even 0.3 μM α_EC could significantly suppress the aggregation of CS. At 37 °C, the aggregation of CS was almost completely suppressed by 0.6 μM α_EC. Similar chaperone-like activity of α_EC was also observed at 30 °C (Figure 4C). These results suggest that the chaperone-like activity of α_EC is temperature dependent and becomes more efficient under physiological temperatures. A similar temperature-dependent property was also reported with sHSPs (*32*,*33*). One possible explanation for this property is that different temperatures may lead to structural changes of the chaperone protein and, in turn, result in different binding capacities to its substrate protein (*33*).

α_EC Can Protect CS from Thermally Induced Inactivation but Inhibit Reactivation of Chemically Denatured CS. CS was readily inactivated upon incubation at 43 °C, during which the inactivation process was monitored by measuring the remaining activity of the enzyme. The effect of α_EC on the thermally induced inactivation process of CS was then tested. At the molar ratio of 1:1 (α_EC:CS subunit), α_EC significantly slowed the inactivation process of CS with an effect comparable to Hsp90 and Hsp27 (Figure 5A). This result indicates that α_EC can interact with partially denatured intermediates of CS during thermally induced unfolding.

However, the effect of α_EC on the reactivation of chemically denatured CS was quite different (Figure 5B). The spontaneous folding reaction of chemically denatured CS was left to occur in HEPES buffer. The yield of reactivated CS after 60 min was about 20%. The reactivation of CS was dramatically increased to as much as about 45%, after it was diluted into a solution of GroEL/S supplied with ATP. However, Hsp27 as well as other reported sHSPs had no effect on the folding kinetics. Interestingly, α_EC significantly suppressed the spontaneous reactivation of CS, and little if any activity was recovered after 60 min. In addition, the suppression could not be affected by ATP. This result indicates that α_EC can form more stable complexes with folding intermediates of chemically denatured CS.

α_EC Cooperates with Other Chaperones To Help Thermally Denatured Luciferase Refold. One of the

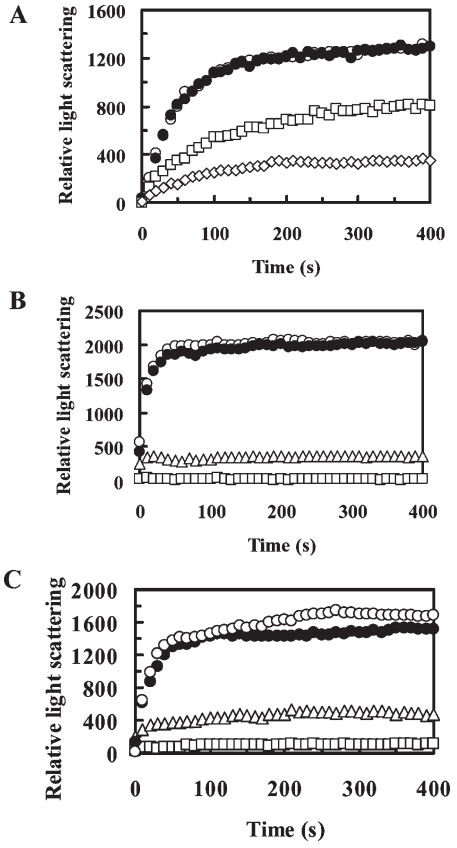

FIGURE 4: Inhibitory effect of α_EC on the aggregation of chemically denatured CS. (A) Chemically denatured CS was diluted into HEPES buffer at 25 °C in the absence (○) or in the presence of 1.2 μM BSA (●), 0.6 μM α_EC (□), and 0.9 μM α_EC (◊). (B) At 37 °C, 0.15 μM CS alone (○) or with 1.2 μM BSA (●), 0.3 μM α_EC (△), and 0.6 μM α_EC (□). (C) At 30 °C, 0.15 μM CS alone (○) or with 1.2 μM BSA (●), 0.3 μM α_EC (△), and 0.6 μM α_EC (□).

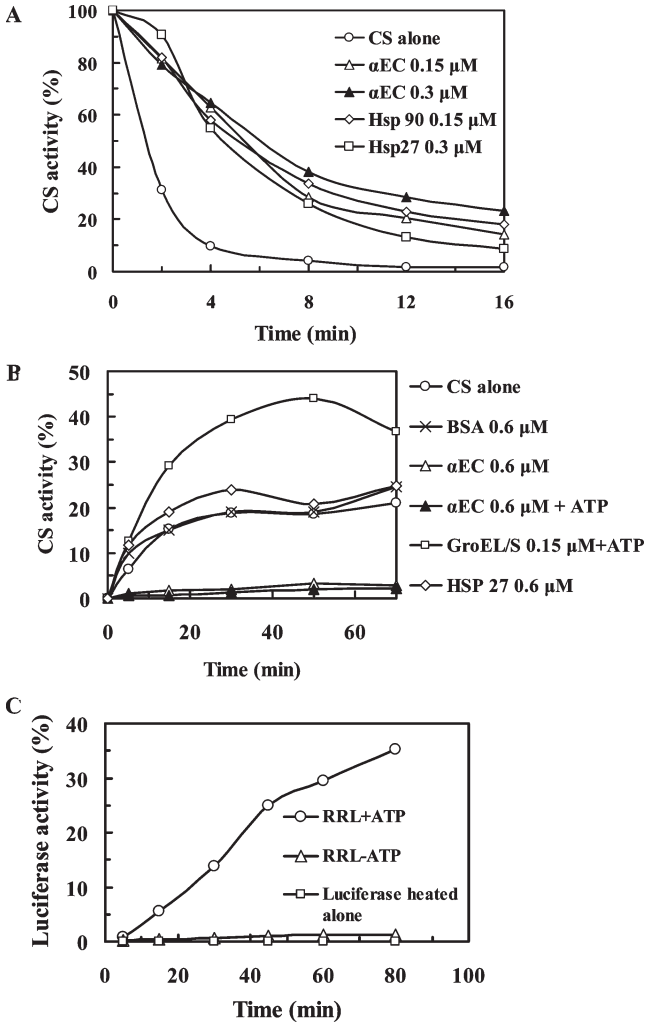

FIGURE 5: The chaperone-like activity of α_EC on CS and luciferase. (A) Thermally induced inactivation of 0.15 μM CS alone (○) or in the presence of 0.15 μM α_EC (△), 0.3 μM α_EC (▲), 0.15 μM Hsp90 (◊), and 0.3 μM Hsp27 (□). (B) Reactivation of 0.15 μM CS alone (○) or in the presence of 0.6 μM BSA (×), 0.6 μM α_EC (△), 0.6 μM α_EC plus ATP (▲), 0.15 μM GroEL/S (□), and 0.6 μM Hsp27 (◊). (C) Heated mixture of luciferase and α_EC added into RRL supplemented with (○) or without ATP (△) or luciferase heated alone and then added into RRL supplemented with ATP (□). The results are representative of two or more individual experiments.

fundamental biochemical properties for molecular chaperones is the ability to maintain non-native proteins in a state that is competent for refolding (*34*). Clusterin, a previously discovered extracellular chaperone, was also reported to have this ability (*35*). To verify this property of α_EC, we used firefly luciferase as a model protein. After coincubation with α_EC at 43 °C, the heated mixture of luciferase and α_EC was then added to 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 (*36*). Reactivation from the heated mixture of luciferase and α_EC reached nearly 35% of the original activity of luciferase before heating (Figure 5C). The reactivation efficiency was comparable to that previously reported (*31*). On the other hand, when luciferase was heated alone in HEPES buffer and then added into RRL, no reactivation was observed (Figure 5C). This result strongly indicates that α_EC can serve as a reservoir, which maintains denatured proteins in a refolding competent state, so that the substrates can later either slowly refold spontaneously or be refolded in conjunction with other chaperones.

α_EC Can Bind with Thermally Stressed Plasma Proteins. To investigate the chaperone-like effect of α_EC on the plasma proteins, we first performed formaldehyde cross-linking to monitor the complex formation process under thermally stressed conditions (*30*). Human plasma with α_EC added was incubated at 37, 43, and 50 °C, respectively. Then formaldehyde cross-linking was performed. As is shown in Figure 6A, HMW complexes appeared after incubation at 50 °C for 30 min, while there was no obvious complex formation at 37 or 43 °C. The amount of α_EC monomer decreased as temperature increased, and almost no α_EC monomer could be detected after incubation at 50 °C (Figure 6A). The result indicates that α_EC may form complexes of HMW with stressed plasma proteins, which is consistent with the result of Figure 3B.

To further specify the substrate proteins of α_EC, we specifically pulled down complexes formed between α_EC and thermally stressed plasma proteins using the polyclonal antibody against α_EC. As is shown in Figure 6B, no additional proteins were pulled down by α_EC at 37 °C, compared with the control without α_EC. However, after incubation at 50 °C for 30 min, there were at least four

additional bands appearing on SDS−PAGE (Figure 6B). Six proteins potentially interacting with α_EC were identified from these four bands by liquid chromatography−tandem mass chromatography (LC-MS/MS), which include human complement component C3, trypsin inhibitor, complement factor B, fibronectin precursor, α-1-microglobulin/bikunin preproprotein, and gelsolin isoform (Table 1). Among these proteins, the existence of proteins belonging to the immune system provides a clue to the situations where the chaperone-like activity of α_EC may be needed. Fibronectin and gelsolin had also been reported to be potentially protected by fibrinogen from thermally induced aggregation (*27*). The identified substrate proteins of α_EC are not totally the same as those of fibrinogen (*27*), possibly due to the different molecule and experimental conditions. The results indicate that α_EC can interact with plasma proteins under stress, preferably by forming HMW complexes. The interactions between α_EC and plasma proteins strongly suggest the biological relevance of α_EC as a chaperone-like protein.

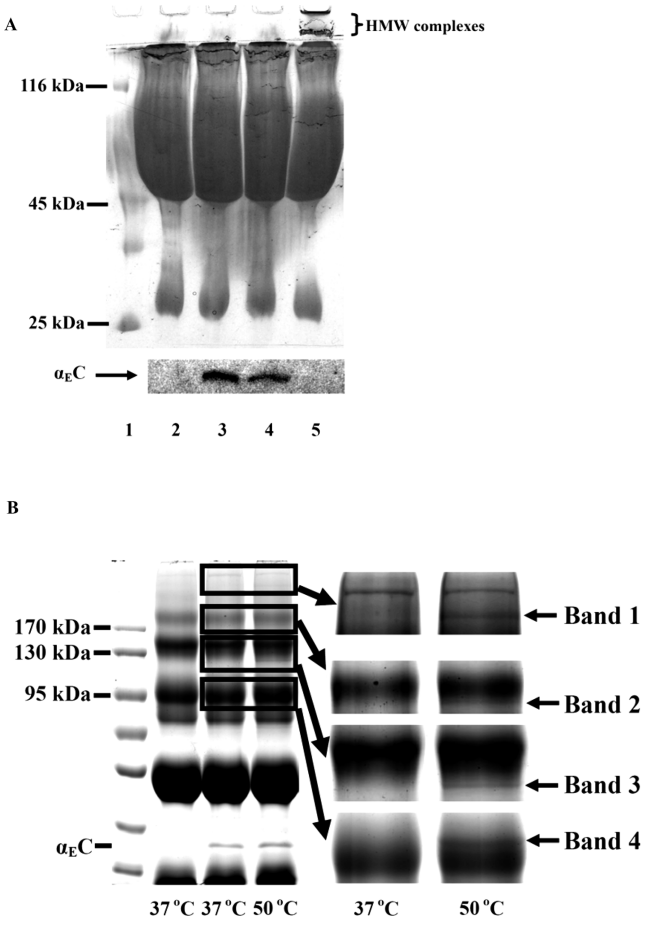

FIGURE 6: α_EC can bind with thermally stressed plasma proteins. (A) Cross-linked protein complexes in the human plasma: lane 1, marker; lane 2, plasma without addition of α_EC at 37 °C; lane 3, plasma added with 2 μM α_EC at 37 °C; lane 4, plasma added with 2 μM α_EC at 43 °C; lane 5, plasma added with 2 μM α_EC at 50 °C. The amount of α_EC was detected with Western blotting. (B) Co-IP of α_EC and thermally stressed plasma proteins: left, SDS−PAGE profile of immunoprecipitated α_EC and plasma proteins; right, amplified region in the rectangular pane.

DISCUSSION

Although the existence of the fibrinogen-420 and α_EC domain was discovered a decade ago, the specific function of α_EC is still largely unknown (*10*,*12*). The effects of α_EC on the aggregation of stressed proteins have not been reported before. Our results presented here show for the first time that α_EC exhibits chaperone-like activity.

The Characteristics of α_EC Are Similar to Those of Small Heat Shock Proteins. α_EC potentially protects a series of proteins such as CS, GAPDH, and MDH from thermally induced aggregation (Figure 2). The efficiency of α_EC is comparable to classic chaperones such as Hsp90 and Hsp27. Also, α_EC can efficiently inhibit aggregation of both thermally denatured and chemically denatured CS protein through forming soluble complexes. As detected by SEC, the complexes formed between α_EC and CS are polydispersed and have high molecular weight (HMW) (Figure 3). Most of the HMW fraction exceeds the exclusion limit of the column (1.3×10^6 Da). sHSPs were known to bind to exposed hydrophobic regions of stressed proteins to form HMW complexes (*37*,*38*). Unlike classic chaperone families such as Hsp70 and Hsp60, α_EC can not refold denatured proteins independently. However, α_EC can keep the partially denatured protein in a folding competent state in the complex and thus help them to refold in cooperation with other chaperone proteins under appropriate conditions (Figure 5C). This property is also well-known for sHSPs (*31*). In addition, the molecular mass of α_EC is 27 kDa, comparable to most of the sHSPs. Taken together, the characteristics of α_EC are quite similar to those of the classic sHSPs. Interestingly, the previously discovered extracellular chaperone-like protein, clusterin, was also reported to be similar to sHSPs in exhibiting chaperone-like activity (*22*).

Table 1: Plasma Proteins Potentially Interacting with α_EC during Thermal Stress[a]

gi number	name of protein	nominal mass (M_r)	score
gi|78101268	human complement component C3	112869	982
gi|33985	trypsin inhibitor	106647	859
gi|291922	complement factor B	85450	660
gi|31397	fibronectin precursor	256529	105
gi|4502067	α-1-microglobulin/bikunin preproprotein	38974	92
gi|4504165	gelsolin isoform	85644	72

[a] For each identified protein, the following information is listed: gi number, used for the records in National Center for Biotechnology Information (NCBI); name of protein, which is generally the same as that used in NCBI data base; nominal mass, which is the measured mass of the protein or protein fragment in LC-MS/MS; score, used for the observed match of LC-MS/MS results with NCBI records. A score > 37 indicates identity or extensive homology ($p < 0.05$) of the observed match, where P is the probability that the result is a random event.

Evolutionary Preservation of the α_E Chain Can Be Explained by the Newly Discovered Chaperone-like Activity. Although fibrinogen-420 and fibrinogen-340 exhibit indistinguishable properties during the coagulation process, the gene encoding the fibrinogen-420 isoform, especially the α_EC domain, is highly conserved among the vertebrates (*9*,*13*,*14*). The cDNA of fibrinogen-420 was first isolated in the chicken genome and then in human beings (*9*,*14*). It contains not only the coding region for the major form of the α chain but also a second open reading frame downstream with high homology to the β and γ chain C-terminal domain. The additional α gene exon (exon VI) encodes a new C-terminus that, when appropriately spliced, extends the α chain (α_E). The basic structure of the α gene is conserved among mammals and birds, as are the intron positions (*9*). Thus, the human α_E isoform and the conventional α chain are identical in sequence starting from residue Val610, but in α_E this valine is followed by Arg611 and the 236 residues encoded by exon VI (Asp612−Gln847) that form the α_EC domain. The sequence of the C-terminal extension of α_E is similar to the carboxyl ends of the β and γ chains. The gene duplications leading to development of the three separate subunits occurred at a similar time, more than 700 million years ago (*39*). The evolutionary preservation of the α_E chains among vertebrates strongly indicates a conserved and specific function of α_EC domain, which can be explained by the newly discovered chaperone-like activity of the α_EC domain.

Structural Basis for the Chaperone-like Activity of α_EC. Structural features are also very important for the chaperone-like activity of α_EC. α_EC is connected to the distal end of the α chain by a long hydrophilic αC tether; therefore, α_EC is spatially more free and consequently interacts more easily with other macromolecules compared with βC or γC. The location of α_EC also ensures more rapid release from the mother molecule given the extreme susceptibility of the αC region to proteolysis (*40*). During fibrin(ogen)olysis, the α_EC domains are released as monomers, unlike the βC and γC domains, which remain anchored together in the proteolytic fragment D.

The Level of α_EC Is Mainly Regulated by Proteolysis. The concentration of fibrinogen-420 has been determined to range from 20 to 150 μg/mL, corresponding to α_EC molar concentrations in the range of 0.1−1 μM (*11*,*41*). Fibrinogen-420 can be more enriched than fibrinogen-340 under certain conditions. For instance, when circulating fibrinogen is taken up by platelets and stored in α granules for release upon platelet activation, fibrinogen-420 is more than twice as enriched in α granules compared with in plasma (*41*). Given the extreme susceptibility of the αC region to proteolysis, the level of α_EC can easily be regulated by the proteases in circulation. The proteolytic degradation makes the generation of free α_EC very rapid under certain stress conditions related with clot formation or fibrinolysis. Matrix metalloproteinases (e.g., MMP3) and trypsin were also found to cleave fibrinogen-420, yielding a final stable degradation product comparable to that generated by plasmin (*41*,*42*). It is not surprising that the local α_EC may be accumulated under some pathological conditions such as myocardial infarction, particularly after thrombolytic therapy, and local levels may reach even higher concentrations, where blood flow is blocked. Notably, accumulation of a free α_EC domain with a half-life of up to 3 h has been observed in the plasma of patients (*12*,*41*). Therefore, it suggests that fibrinogen-420 may have evolved for the purpose of delivering free α_EC as a vehicle in addition to its clotting activity. Recently, a growing number of comparable proteolytic products exhibit potent effects unrelated to the primary function of their parent molecules, which often serve to release fragments to sites of tissue repair, wound healing, and angiogenesis (*43*). To our knowledge, α_EC may be the first discovered chaperone-like protein generated by proteolytic degradation.

Biological Significance. We discovered that α_EC can specifically interact with and suppress the aggregation of a wide spectrum of stressed proteins (Figures 2−4). Furthermore, α_EC can interact with different folding intermediates either on the thermally induced unfolding pathway or on the refolding pathway of chemically unfolded CS (Figure 5) (*29*). The general effect of α_EC on stressed proteins is also supported by the fact that α_EC can bind with thermally stressed plasma proteins and form soluble complexes (Figure 6). It is understandable that the potential substrates of α_EC under stress conditions are including but not limited to the proteins that we have identified (Figure 6).

Coagulation, fibrinolysis, and inflammation process are closely related (*44*). Oxidative stress is well-known to be induced by inflammation responses (*45*), which may lead to protein misfolding or tissue damage. As an acute phase protein, the expression of fibrinogen is induced by interleukin 6 (IL-6) (*3*), while the release of α_EC is mainly regulated by plasmin or trypsin (*12*,*42*). It is highly possible that the chaperone-like activity of fibrinogen/α_EC would play an important role under stress conditions relating to inflammation, clot formation, or fibrinolysis.

Substrates in complexes with α_EC may be refolded when the stress conditions disappear (Figure 5) or cleared by other extracellular quality control mechanisms. Previous studies have indicated that 50−90% of plasma proteins are degraded in the liver and reticuloendothelial system (*46*). It was also reported that α_EC binds to integrins on the surface of the leukocyte (*20*). Therefore, α_EC may be able to facilitate the cellular uptake and degradation of misfolded proteins to which it binds or recognize damaged proteins to mediate the immune response under stress conditions.

It would be very interesting to elucidate the relation between α_EC and amyloid diseases. The other previously reported extracellular protein clusterin has been reported to be able to inhibit the amyloid fibril formation process of lysozyme (*47*). Interestingly, an American kindred was reported to have a single nucleotide deletion at the third base of codon 524 of the fibrinogen α chain gene that resulted in a frame shift and premature termination of the protein at residue 548 (*48*). As a result, the C-terminal segment 525−625 of the common α chain was lacking, and formation of α_E was impossible. Symptoms displayed were hereditary renal amyloidosis and low plasma fibrinogen concentration. Further analysis of the chaperone-like function of α_EC under these pathological conditions may provide new clarification in this regard.

Taken together, the α_EC domain of fibrinogen-420 exhibits a chaperone-like activity similar to that of sHSPs. Unlike classic chaperone proteins, α_EC is an extracellular protein in the circulatory system, which is mainly regulated by proteolytic degradation. Our findings not only provide new insights into the novel function of α_EC but also have implications on potential diagnostic and therapeutic approaches to many pathological conditions.

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