Capacity of Human Serum to Depolymerize Actin Filaments

By Paul A. Janmey and Stuart E. Lind

Human blood depolymerizes filamentous (F-) actin. The interaction of actin filaments and monomers with human serum was studied by following the kinetics and extent of the depolymerization of pyrene-labeled F-actin and by analysis of serum proteins adhering to immobilized actin monomers. In physiologic Ca\(^{2+}\) concentrations, the depolymerization of F-actin proceeds in two stages: a rapid phase, attributed to direct severing of filaments by plasma gelsolin, and a slow phase attributed to the binding of actin monomers to vitamin D-binding protein (DBP). Without Ca\(^{2+}\), only the slow phase is observed. Human serum can completely depolymerize 10 to 18 μmol/L of actin, of which ~5 μmol/L occurs rapidly. Depolymerization can be accounted for by the normal serum concentrations of gelsolin and DBP. Fibrin(ogen) and fibronectin, which bind actin in vitro, do not contribute to the kinetics or extent of its depolymerization. Affinity chromatography and functional assays for the presence of gelsolin–actin complexes show that addition of G-actin to serum results in preferential formation of actin–DBP complexes, but that addition of F-actin to serum produces both gelsolin–actin complexes and DBP–actin complexes. The distinctive binding of actin monomers and polymers to these two serum proteins suggests a means by which their coordinated actions are maximized in vivo, from the standpoint of depolymerizing filaments and clearing monomers from the circulation.

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MATERIALS AND METHODS

Materials. DNAase I was purchased from Sigma Chemical (St Louis). CNBr-activated Sepharose 4B (Pharmacia, Piscataway, NJ) was coupled to DNAase I (1 mg/mL of hydrated beads) according to the manufacturer’s instructions. N-(1-pyrenyl)-iodoacetamide was obtained from Molecular Probes (Eugene, OR).

Serum preparation. Serum was prepared by allowing blood to clot in a glass tube for 2 hours at 37 °C and then centrifuging it for 20 minutes at 3,000 g. Plasma was prepared from ACD-anticoagulated blood supplied by the blood bank and was centrifuged twice at 3,000 g for 20 minutes in plastic containers to remove cellular elements.

Protein purification. Gelsolin was purified from plasma by monclonal anti-gelsolin antibody affinity chromatography by the method of Chaponnier and colleagues. DBP was prepared from plasma by the method of Haddad and associates. Following passage of the plasma over a column of Sepharose 4B coupled to anti-gelsolin antibody. DBP was prepared from rabbit skeletal muscle and labeled with N-(1-pyrenyl)-iodoacetamide as described elsewhere.

Pyrene-labeled actin was stored frozen in liquid nitrogen as monomeric (G-) actin in 2 mmol/L of Trishydroxymethyl-aminomethane (Tris), 0.2 mmol/L of ATP, 0.2 mmol/L of CaCl\(_2\), 0.2 mmol/L of 2-mercapto-ethanol, pH 7.8. It was diluted 1 day before measurements were made and mixed with various amounts of unlabeled actin.

Functional assays for serum gelsolin concentration. The total gelsolin concentration (free or bound to actin) of serum or plasma samples was determined from their ability to accelerate actin assembly. The concentration of free gelsolin (ie, not bound to actin monomers) was determined by measuring the filament-severing ability of the serum. The assay is based on the fact that when pyrene-labeled actin filaments are severed by gelsolin and then diluted below the critical concentration of the slow-exchanging (–)
filament end (0.5 to 1.2 μmol/L), the rate at which such filaments depolymerize is proportional to the number of (-) ends, which is equal to the number of cuts made by gelsolin. Previous experiments have shown that preincubation of purified gelsolin with actin inhibits its severing activity such that nearly all filament-severing activity is lost at a molar ratio of actin to gelsolin of 2:1.

Measurement of actin depolymerization by serum. Depolymerization of F-actin by serum samples was measured by first adding pyrene-labeled actin filaments (polymerized by addition of 2 mmol/L of MgCl₂ and 150 mmol/L of KCl) to the sample in a cylindrical glass light-scattering tube (6-mm diameter, 200-μL sample volume), gently mixing the solution by drawing it up in a Pasteur pipette, and then placing the scattering tube in a fluorometer (Perkin Elmer LS-5, Oak Brook, IL). The time between mixing and the first fluorescence measurement was 5 to 10 seconds. The actin content of some samples was varied by altering the concentration of unlabeled actin, keeping the pyrene–actin concentration constant prior to polymerization with added salts. Pyrene-labeled actin copolymerizes randomly with unlabeled monomers under these conditions. Some serum samples were dialyzed into buffers containing 50 mmol/L of Tris and 150 mmol/L of NaCl (TBS) to assess the possible effects of dialyzable factors, especially Ca²⁺. Some serum samples were depleted of gelsolin by passing 4 mL of serum over a 1.5-mL column of Sepharose-coupled anti-gelsolin antibody equilibrated with TBS plus 3 mmol/L CaCl₂ (TBS–Ca²⁺). Control whole serum was diluted to an equal total protein concentration.

Affinity chromatographic assay for actin binding. Varying amounts of G-actin were added to serum. After a 10-minute incubation at 23 °C, the serum–actin mixture was added to 40 μL of DNAase I immobilized on Sepharose 4B beads. The mixture was incubated with DNAase beads for 2 hours at 4 °C with continuous end-over-end tumbling. The DNAase beads were sedimented by centrifugation at 18,000 g for 1 minute and washed once in TBS–Ca²⁺ with 0.75% Triton X-100 (Sigma, St Louis) and twice with TBS–Ca²⁺. Proteins adhering to the DNAase beads were eluted with the gel sample buffer of Laemmli, analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and their mol wt was compared to known standards. The amounts of DBP and gelsolin bound to actin were quantified by scanning Coomassie blue-stained bands with a laser scanning device and multiplying the relative areas by the ratios of mol wt of actin (42 kd), DBP (56, kd), and gelsolin (93 kd).

RESULTS

Kinetics of actin depolymerization by serum with and without Ca²⁺. The depolymerization of F-actin by serum depends on the free Ca²⁺ concentration, as has been reported previously by measurements using other assays. As shown in Fig 1, depolymerization of F-actin added to whole serum proceeded rapidly. Removal of Ca²⁺ severely retarded the initial rate of depolymerization and its final extent. Addition of Ca²⁺ to depleted samples restored the rate to that in whole serum. The depolymerization in Ca²⁺ proceeds in two stages: a rapid initial phase that is completed in several seconds followed by a slower phase that occurs over a period of many minutes. Without Ca²⁺, the initial rapid phase of depolymerization does not occur. Instead, a slow steady decrease in F-actin fluorescence occurs, leading, after several hours, to partial depolymerization.

Capacity of serum to depolymerize actin. Various amounts of actin were added to serum (in the presence of Ca²⁺) to determine the filament-depolymerizing capacity of serum. Depolymerizing activity was resolved into slow and rapid phases by monitoring the amount of filamentous actin present 30 seconds and 7 hours after addition of serum (Fig 2). The two phases were saturated at different actin concentrations. The slow phase of depolymerizing activity was saturated at three to four times the actin concentration measured by nucleation assays was 0.74 μmol/L.
required to saturate the rapid phase. The fraction of total depolymerization that occurs during the initial phase is a function of the amount of F-actin added to the serum. Measurement of the gelsolin concentration of the specimen showed that the rapid phase was saturated at a ratio of actin to gelsolin of 2:1. The relative extents of the fast and slow depolymerization phases as a function of the added actin concentration are shown in Fig 2, which shows the fluorescence of pyrene-labeled actin added to a serum solution containing 0.69 μmol/L of gelsolin measured 30 seconds and 7 hours after the addition of F-actin samples containing a constant amount of pyrene-labeled actin copolymerized with different amounts of unlabeled actin. When the total actin concentration was <1.7 μmol/L, nearly all the fluorescence was eliminated in 30 seconds, suggesting that when a molar ratio of <2:1 of actin to gelsolin exists, depolymerization proceeds rapidly. When 4 μmol/L of total actin was added, nearly 90% of the initial fluorescence was observed after 30 seconds, but the fluorescence decrease was nearly total after 7 hours, suggesting that the actin eventually depolymerized by a different mechanism. When the final actin concentration was >10 μmol/L, the fluorescence was only partially decreased even after 7 hours, suggesting that most of the actin remained in polymeric form. Both the fast and slow phases of depolymerization are saturable. The rapid phase was saturated when the ratio of actin to gelsolin was ~2:1, but saturation of the slow phase of depolymerization was observed at approximately threefold higher actin concentrations.

Effect of low concentrations of F-actin on the filament-severing and polymerization-accelerating activities of serum. The severing activities of serum samples (total gelsolin concentration 0.7 μmol/L) containing different concentrations of unlabeled F-actin are shown in Fig 3. The free gelsolin concentration (severing activity of the serum) decreased in direct proportion to the amount of unlabeled F-actin added during the preincubation step, with nearly total loss of severing activity at a concentration of 1.5 μmol/of actin, a molar ratio of nearly 2:1 with respect to gelsolin. Although such a serum sample could not sever actin filaments, it had depolymerizing activity since, as shown in Fig 2, nearly 5 μmol/L of actin was totally depolymerized by serum samples after several hours. This result suggests that the slow phase of depolymerization is due to a component of serum that does not sever actin filaments. In contrast to their effects on the filament-severing activity of serum, the presence of these concentrations of F-actin had no effect on the ability of serum to nucleate actin assembly from monomers, as shown in Fig 3. This result is characteristic of the effects of actin on purified gelsolin.13

Different effects of G-actin and F-actin on the depolymerizing properties of serum and mixtures of gelsolin and DBP. If G-actin rather than F-actin was added to serum, the severing activity of the serum was not diminished, as shown in Fig 4. The initial rapid phase of depolymerization was eliminated by prior incubation of serum with F-actin. In contrast, when an equivalent amount of G-actin was added, the rapid phase of depolymerization was nearly the same as that which occurred with control serum.
merization of F-actin by the purified proteins was not affected by the addition of physiologic concentrations of fibrinogen or fibronectin, (data not shown).

Preferential binding of G-actin to DBP in the presence of free gelsolin. G-actin was added to serum and then isolated together with bound proteins from the mixture with DNAase-Sepharose. (DNAase I has a high affinity for G-actin and forms ternary complexes containing DNAase–actin–DBP or DNAase–actin–gelsolin.) As shown in Fig 7B, polypeptides of mol wt 56,000, 66,000, and 93,000 were observed when G-actin was added to serum. The 93,000 polypeptide comigrates with gelsolin, immunoprecipitated from the same sample by immobilized monoclonal anti-gelsolin antibodies (lane A). The 56,000 polypeptide is presumed to be DBP. These two polypeptides are bound to actin, since they are not precipitated by DNAase beads alone. The 66,000 polypeptide is presumed to be albumin. Its binding appears to be nonspecific, because this band was also observed for samples prepared from serum without added G-actin, and the amount of this polypeptide did not generally correlate with the amount of added actin. The amounts of DBP and gelsolin that coprecipitated with various concentrations of actin are shown in Fig 7A. For these samples, which contained 50% serum by volume, an equimolar amount of DBP was bound to actin at actin concentrations up to 4 μmol/L and binding appeared to be saturated at 6 μmol/L of DBP. Almost no gelsolin was apparent when <4 μmol/L G-actin was added to the samples; thereafter the amount of gelsolin increased. This finding confirms that G-actin binds preferentially to DBP, and when this binding is complete, actin monomers bind also to gelsolin.

**DISCUSSION**

Undiluted serum can depolymerize 10 to 18 μmol/L of F-actin. Of this total, ~5 μmol/L is depolymerized immedi-
Fig 7. Isolation of G-actin-containing complexes from serum. (A) Increasing amounts of G-actin were added to mixtures of 100 μL of serum and 100 μmol/L of 50 mmol/L of Tris, 150 mmol/L of NaCl (TBS). Actin-containing complexes were isolated with DNase-Sepharose beads. After washing, the beads were incubated with gel sample buffer and loaded on 5% to 15% minigels, and the gels were stained with Coomassie blue. The amount of gelsolin (closed symbols) and vitamin D-binding protein (DBP) (open symbols) coprecipitating with different amounts of actin were determined from densitometry of Coomassie-stained bands. Different symbols represent separate experiments. (B) Representative lanes are shown for proteins adhering to DNase beads added to serum containing 0.5 (a), 1.5 (b), 3.0 (c), or 7 (d) μmol/L of G-actin. Lane : Serum proteins bound to immobilized anti-gelsolin antibodies.

Because the depolymerization of actin filaments by serum is reproduced by solutions containing physiologic concentrations of purified gelsolin and DBP, these two proteins account for most of the actin-depolymerizing action of serum, with a minor additional effect contributed by calcium ions.

Serum gelsolin and DBP function in a coordinated manner to maximize their actin-depolymerizing properties. If serum contained only gelsolin, it would lose its filament-severing capacity as soon as actin appeared extracellularly, even if only actin monomers were released. The presence of DBP, to which actin monomers bind preferentially, ensures that gelsolin will not become saturated with actin monomers, but will be able to sever actin filaments. Thus, DBP prevents the loss of the rapid filament-severing activity of serum due to release of G-actin. The preservation of gelsolin’s filament-severing capacity complements DBP’s role as the major actin-clearing protein of blood.

The significance of serum actin-depolymerizing proteins has become more apparent in the light of recent evidence of actin in the extracellular space. The difficulty of documenting the presence of actin in the blood may be due to the efficiency of the proteins that sequester and clear it (gelsolin and DBP). Alternatively, its affinity for fibrin may result in its being inadvertently discarded with the fibrin clot when serum is prepared.

Fig 8. Schematic of complexes formed following addition of G-actin or F-actin to gelsolin and vitamin D-binding protein (DBP). Diagrams indicate the different mechanisms by which complexes are formed when actin filaments or monomers are added to a solution containing gelsolin and DBP at their appropriate molar ratio in human blood. When F-actin is added, complexes form first with gelsolin and secondarily with DBP. If an equivalent amount of G-actin is added, complexes form preferentially with DBP. Most of the gelsolin remains free and capable of severing actin filaments.
ACTIN FILAMENT DISASSEMBLY IN SERUM

The combined effects of gelsolin and DBP (at their physiologic molar ratios) on G-actin and F-actin are summarized in Fig 8. When F-actin is added to serum, it binds first to gelsolin to form filament fragments that have a gelsolin cap on the (+)-end. DBP can then remove actin monomers from these complexes, forming 1:1 actin–DBP complexes, until all the DBP is complexed. If less than ~15 μmol/L of actin is added (ie, the amount of F-actin that can be totally depolymerized), 1:1 actin–gelsolin complexes will remain as the DBP–actin complexes form. If instead, the same amount of G-actin is added, the actin monomers bind first to DBP. When all DBP has formed 1:1 complexes with actin, the remaining actin monomers bind to gelsolin. Because the binding of actin to gelsolin is cooperative, the serum will contain a mixture of 2:1 actin–gelsolin complexes and free gelsolin, but not 1:1 actin–gelsolin complexes.

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REFERENCES

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PA Janmey and SE Lind