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# SEQUENTIAL BINDING OF ACTIN MONOMERS TO PLASMA GELSOLIN AND ITS INHIBITION BY VITAMIN D-BINDING PROTEIN

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**Functional studies that distinguish free from actin-bound gelsolin based on the ability of the former to sever actin filaments reveal that the binding of actin monomers to gelsolin is highly cooperative and can be prevented by prior incubation of actin with vitamin D-binding protein (DBP), even though the apparent affinity of gelsolin for actin is 50-fold greater than that of DBP. Measurements of actin binding by immunoprecipitation and pyrene-actin fluorescence establish that DBP-actin complexes do not bind to gelsolin and that DBP removes one of the actin monomers in a 2:1 actin-gelsolin complex. These studies may explain why DBP-actin complexes exist in blood plasma in vivo in the presence of free gelsolin and suggest that the interaction of gelsolin with actin in cells and plasma may be regulated in part by actin monomer binding proteins.**


Introduction - Plasma gelsolin (also called brevin) (1-4) and vitamin D-binding protein (DBP)(5,6) are the two major actin binding proteins of mammalian blood . DBP depolymerizes actin filaments (F-actin) by sequestering actin monomers (G-actin) that exchange with the ends of actin filaments. Gelsolin actively severs F-actin and, at high gelsolin:actin ratios, forms ternary complexes with two actin monomers. Plasma gelsolin, which is closely related to cytoplasmic gelsolin, also differs from DBP in that its function requires the presence of $Ca^{2+}$. Plasma gelsolin and DBP may serve an important physiologic role by either disassembling long actin filaments released into the bloodstream following tissue injury (7,8) or accelerating the clearance of actin from the circulation (9). Previous studies have shown that gelsolin and DBP can act independently of each other and depolymerize F-actin at different rates (10,11). Whether they compete with each other for binding to actin or

Abbreviations used: DBP, Vitamin D-binding protein; EGTA, Ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid.

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bind to different sites on the actin monomer is not known. Binding studies of purified complexes have shown that the affinity of DBP for actin ($K_a$) is 1.8 x $10^7$ $M^{-1}$ (12) and the affinity of gelsolin for actin is $10^9$ $M^{-1}$ (13). These values suggest that in the presence of gelsolin, very little actin would bind to DBP. However, in vivo studies suggest that appreciable amounts of DBP-actin complexes are formed under conditions where free gelsolin is present (9,14). Gelsolin has been shown to possess two separate actin binding sites, which differ in structure and binding properties (15). The evidence presented here shows that DBP can compete with the one of these sites for the initial binding to actin and thereby, under some circumstances compete successfully with gelsolin for actin.

## Materials and Methods

Proteins - Actin was purified from rabbit skeletal muscle by the method of Spudich and Watt (16) and labeled with N-(1-pyrenyl)- iodoacetamide (17) a fluorescent adduct that increases fluorescence when actin polymerizes. Pyrene-labeled actin was mixed with unlabeled actin under depolymerizing conditions (2 mM Tris, 0.2 mM ATP, 0.2 mM $CaCl_2$, 0.2 mM 2-mercaptoethanol, pH 7.8 : Buffer A) and further purified by gel permeation chromatography on G-150 Sephadex (Pharmacia). Gelsolin was purified from human plasma by monoclonal anti-gelsolin antibody affinity chromatography (18). DBP was prepared by the method of Haddad et al. (19) with the modification that plasma was first depleted of gelsolin by affinity chromatography as described above (18). Antibody to DBP was prepared in goats immunized with purified human DBP by a method previously described (20). The anti-DBP antibody was affinity purified by passage over a column of human DBP coupled to CNBr-activated Sepharose 4B and eluted by 0.58% acetic acid. Protein concentrations were determined by the method of Bradford (21) with bovine IgG as the standard.
Functional assay for free gelsolin. - When added to preformed actin filaments, gelsolin severs the filaments and remains bound to their fast-growing (+) ends. When such filaments are diluted below the critical concentration of the slow-growing (-) end, the filaments depolymerize at a rate proportional to the number of ends, which is approximately equal to the concentration of free gelsolin originally added (22,23). Gelsolin that is bound to 1 or 2 actin monomers binds to free (+) ends but does not sever filaments, and does not therefore accelerate depolymerization (11,24). To determine the concentration of unbound gelsolin in mixtures containing gelsolin, actin, and DBP, 10 ul of sample were added to 10 ul of 12 uM pyrene-labeled F-actin and the mixture was diluted to a final pyrene-actin concentration of 0.3 uM in buffer A with 2 mM $MgCl_2$ and 150 mM KCl (buffer B). Fluorescence was measured using an excitation wavelength of 365 nm and an emission wavelength of 385 nm with a slit width of 3 nm. The rate of its decrease is related to the rate of actin depolymerization (23) which is related to the gelsolin concentration by the expression $-d[F\text{-actin}]/dt = k_- [g]$, where [g] is the concentration of gelsolin that was not already bound to actin monomers prior to addition to pyrene-labeled F-actin and $k_-$ is a constant equal to 0.03/s under these conditions (23).

Predictions of the amount of free gelsolin in the presence of different actin concentration are calculated for three possible binding schemes. If the two actin binding sites are independent and of equal affinity, and only one

actin is needed to eliminate severing ability, the the fraction of gelsolin that retains severing activity is given by

$$f_1 = (1-p)^2 \quad (1)$$

where p is the probability that an actin binding site is occupied.
If binding is independent and both sites need to be occupied to block severing, the fraction of functional gelsolin is given by:

$$f_2 = 1 - p^2 \quad (2)$$

A third mechanism is one in which binding is strongly cooperative, so that the binding of the first actin monomer to gelsolin greatly increases the probability of occupation of the second actin binding site. In this case the fraction of gelsolin that can sever is

$$f_3 = 1 - p \quad (3)$$

Fluorescence measurements of gelsolin-actin complex formation - The fluorescence of pyrene-labeled actin monomers at 386 nm following excitation at 365 nm is enhanced following binding of either DBP or gelsolin, but gelsolin has a far greater effect (10,25). Competition between DBP and gelsolin was studied by adding DBP to gelsolin-actin complexes, and by adding gelsolin to DBP-actin complexes in buffer A.
Affinity chromatography - A solution of gelsolin (1 uM) and actin (2 uM) was prepared in buffer B. After a ten minute incubation at 23°C, 2 uM DBP was added to half of the sample and then incubated for an additional ten minutes. At that time 50 ul of each sample were added to 20 ul of packed Sepharose 4B beads coupled to either polyclonal anti-DBP antibody or monoclonal anti-gelsolin antibody. After a 30 minute incubation the mixtures were centrifuged for 2 minutes at 18,000 x g, and the pellet was washed once with 1 ml of buffer B. The entire pellet and supernatant were loaded onto 5-15% SDS-polyacrylamide gels for analysis of polypeptide composition by electrophoresis (26). The gels were stained with Coomassie blue dye and the relative amounts of proteins of interest in the pellet and supernatant were quantified by densitometry.

## Results

Cooperative formation of actin-gelsolin complexes - The severing ability of gelsolin (proportional to the amount of free gelsolin) previously incubated with various amounts of G-actin is a function of the actin:gelsolin ratio as shown in Figure 1. The severing function of gelsolin decreases in direct proportion to the amount of actin with which it was incubated before addition to actin filaments. A complete loss of severing function is seen at an actin: gelsolin ratio of 2:1. Figure 1 also shows that the data are best fit to the prediction of Equation 3 indicating cooperative binding of actin to gelsolin. The second actin therefore binds to gelsolin more avidly than the first.

Gelsolin severing function in the presence of actin and DBP - If DBP were to compete with gelsolin for binding to actin monomers, a higher total actin concentration would be required to eliminate the severing function of gelsolin

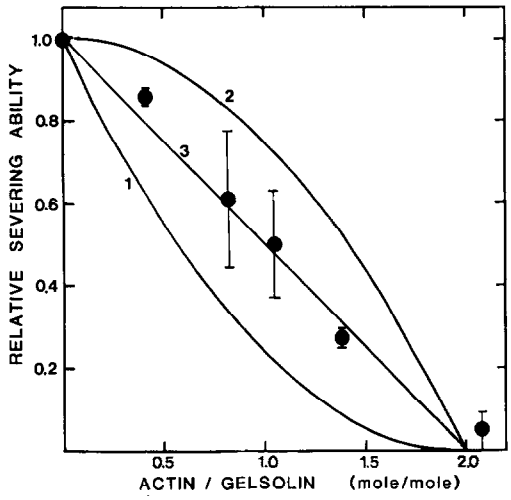


Figure 1. **Elimination of gelsolin severing function by prior incubation with actin.** 0.65 uM gelsolin was incubated with various amounts of actin prior to addition to pyrene-labeled actin filaments. After ten minutes incubation in buffer A plus 75 mM KCl, the gelsolin-actin mixtures were added to 20 parts buffer B with simultaneous addition of pyrene-labeled F-actin to a final concentration of 290 nM. The depolymerization rate (mean +/- standard deviation for measurements performed in duplicate or triplicate indicated by the points) was monitored by the decrease of fluorescence. The theoretical curves are calculated from the corresponding equations in the text.

in the presence of DBP than in its absence. This prediction is verified by the data shown in Figure 2. When an equimolar amount of DBP was added to G-actin prior to the addition of gelsolin, the gelsolin retained its full severing

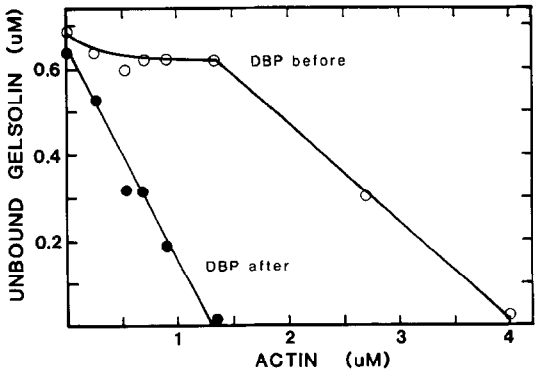


Figure 2. **Effects of DBP on severing function of gelsolin preincubated with actin.** 2.1 uM DBP was added to various amounts of actin either 5 minutes before ( O ) or 10 minutes after ( ● ) addition of 0.65 uM gelsolin to actin. After an additional 30 minutes, the solutions containing all three proteins were added to pyrene-labeled F-actin prior to depolymerization as described for Figure 1. Nearly identical results were obtained when the incubation period was 16 hrs prior to addition to F-actin. The line drawn through the solid circles is the least squares fit to the data of Figure 1 for gelsolin-actin complexes without DBP.

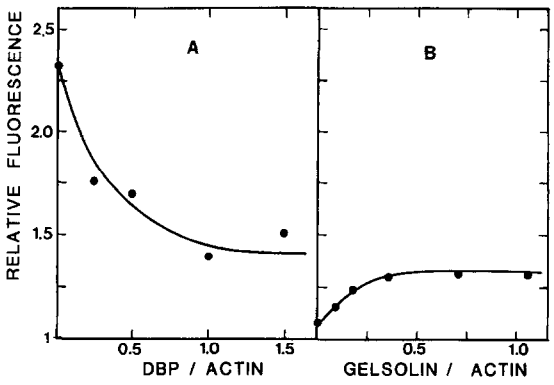


Figure 3. **A. Effect of DBP on fluorescence of pyrene-labeled actin bound to gelsolin.** 0.38 uM G-actin was incubated with 0.14 uM gelsolin in buffer A. After 10 minutes, DBP was added to various molar ratios to actin. Fluorescence was measured 10 minutes later. Relative fluorescence is the ratio of sample fluorescence to that of actin alone under the same ionic conditions, with a background signal from buffer alone subtracted from all measurements. **B. Effect of gelsolin on fluorescence of pyrene-labeled actin bound to DBP.** 0.38 uM G-actin was incubated with 0.38 uM DBP prior to addition of gelsolin to various molar ratios to actin. Conditions are as given in Figure 3A.

function, even after 16 hours of incubation. This finding suggests that the first actin binds to gelsolin more weakly than to DBP. However, when actin-gelsolin complexes were formed before DBP was added, the severing function of gelsolin was not restored, even if an excess of DBP was added, and the incubation prolonged for 16 hours. Therefore, at least one of the actins in the ternary complex cannot be removed by DBP.

Fluorescence measurements of competitive binding - The effect of adding DBP to gelsolin-actin complexes is shown in Figure 3a. The fluorescence enhancement of the gelsolin-actin complex is partially decreased by DBP in a concentration-dependent manner that reaches saturation, suggesting that DBP removes one actin from the 2:1 actin-gelsolin complex. Figure 3b shows that addition of gelsolin to preformed actin:DBP complexes does not result in the fluorescence enhancement seen when gelsolin is added to actin in the absence of DBP.

Affinity chromatography - Solutions containing a 2:1 molar ratio of actin to gelsolin were added to DBP (in amounts equimolar to actin) prior to immunoprecipitation. As shown in Figure 4, gelsolin-actin complexes did not adhere to anti-DBP beads; only 12% of the gelsolin and 10% of the actin cosedimented with anti-DBP antibodies. When DBP was added to gelsolin-actin complexes, 83% of the actin cosedimented with anti-DBP, as did 95% of the DBP.

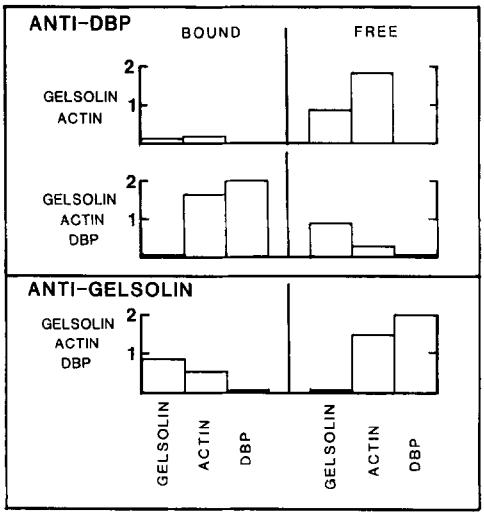


Figure 4. **Isolation of actin-containing complexes with affinity chromatography using antibodies to gelsolin and DBP.** The bars depict the relative molar amounts of gelsolin, actin, and DBP bound and unbound to immobilized antibodies directed against DBP (upper panel) or gelsolin (lower panel). The molar ratio of gelsolin:actin:DBP in the starting mixtures was 1:2:2. Reaction conditions as given in the text.

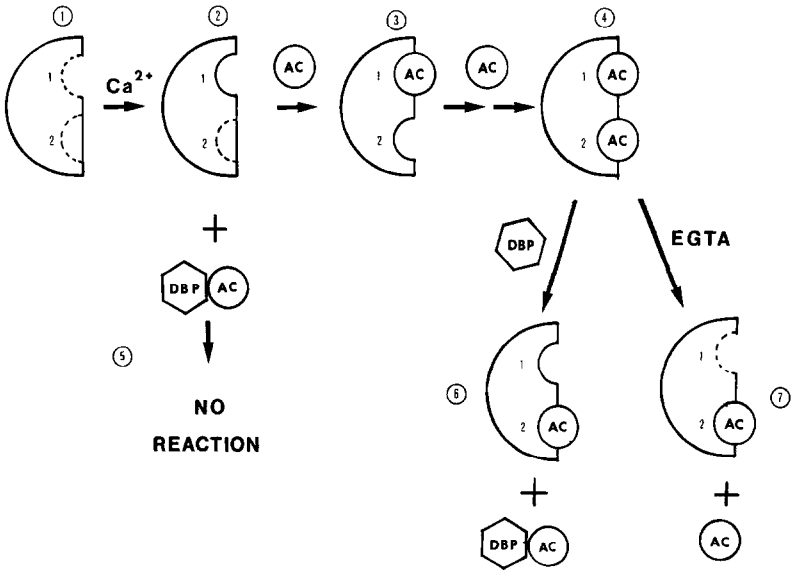


Figure 5. **Schematic summary of the binding of actin monomers to gelsolin and the effect of DBP.** $Ca^{2+}$ exposes one actin binding site on gelsolin. DBP-actin complexes cannot bind to gelsolin at this stage, but free actin monomers bind to gelsolin and thereby expose a second actin binding site (3). Binding of actin monomers to this second site is of higher apparent affinity than binding to the first site. Free $DBP_{2+}$ can remove an actin monomer from site 1 but not site 2 (6). Chelation of $Ca^{2+}$ by EGTA also releases the actin monomer bound to site 1.

In contrast, 85% of the gelsolin cosedimented with immobilized anti-gelsolin antibodies, along with a nearly equimolar amount (26%) of the actin, but less than 5% of the DBP. These direct binding data support the functional results indicating that: 1) DBP can remove one of the two actins bound to gelsolin in $Ca^{2+}$ and 2) that a ternary complex containing actin-gelsolin-DBP is not formed. The sequence of binding of actin monomers to gelsolin and the effect of DBP are summarized in Figure 5.

Discussion - The positive cooperativity of the two separate actin-binding sites of gelsolin helps to explain the findings that although DBP has a 50 fold lower affinity for actin than does gelsolin, appreciable amounts of DBP-actin complexes are found in vivo under conditions where free gelsolin is present. DBP can successfully compete with the binding of the first actin to gelsolin and thereby prevent the formation of the high-affinity 2:1 complex. Once this complex is formed, DBP can remove one of the actin monomers from the complex, leaving the higher-affinity $Ca^{2+}$- irreversible binding intact. One possible consequence for this competition in vivo may be related to the specialized actin modulating effects of these two plasma proteins. When a mixture of monomeric and polymeric actin is released as a result of tissue injury, for example, monomers would preferentially react with DBP, leaving gelsolin free to sever actin polymers, thereby increasing the number of filament ends and further accelerating depolymerization. Since depolymerization of F-actin by DBP alone is a slow process, such filament severing may be essential for efficient filament disassembly.

In addition to its role in blood, gelsolin is a major actin associated protein of the cytoplasm of eukaryotic cells (20,27). Although DBP has not been detected in cytoplasm, the protein profilin, which has functional properties similar to those of DBP (28) is probably the most abundant cytoplasmic actin associated protein. Therefore, the effects of the monomer sequestering protein DBP on the binding of actin to gelsolin may also be relevant to the function of cytoplasmic gelsolin. The cooperative binding of actin to gelsolin suggests that the interaction of gelsolin with both G- and F-actin may be closely regulated by the interaction of actin with other actin

associated proteins, such as profilin, even if such proteins have a lower apparent affinity for actin than does gelsolin.

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