Immuno-identification of Ca\textsuperscript{2+}-induced Conformational Changes in Human Gelsolin and Brevin

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Abstract. Gelsolin is a 90,000-mol-wt protein with two actin and two high affinity calcium-binding sites that can form complexes with Ca\textsuperscript{2+} ions and monomeric actin. These complexes will nucleate filament growth and cap the barbed end of filaments, but will not fragment F-actin. Uncomplexed gelsolin sever F-actin. (Bryan, J., and L. M. Coluccio, 1985, J. Cell Biol., 101:1236-1244). These associations with actin are modulated by Ca\textsuperscript{2+}. We have purified and characterized monoclonal antibodies that recognize Ca\textsuperscript{2+}-induced conformational changes in human platelet gelsolin (G) and human plasma brevin (B), a closely related protein. Two hybridomas, 8G5 and 4F8, were adapted to growth in serum-free medium. 8G5 was found to secrete an IgG; 4F8 secretes an IgA. On immunoblots, both antibodies gave a strong reaction if Ca\textsuperscript{2+} was present, but gave barely detectable reactions if EGTA was used. 8G5 IgG-Sepharose columns retained gelsolin (as GCa\textsubscript{2}) or brevin (as BCa\textsubscript{2}) in 0.1 mM CaCl\textsubscript{2} containing buffers, but released these molecules when eluted with 4 mM EGTA. 8G5 IgG-Sepharose columns also retained gelsolin-actin-Ca\textsuperscript{2+} complexes, as GA\textsubscript{1}Ca\textsubscript{2} or higher oligomers from platelet extracts containing 0.1 mM CaCl\textsubscript{2}. Elution with 4 mM EGTA released material that gel filtration showed to be the EGTA-stable 130,000-mol-wt gelsolin-actin complex, GA\textsubscript{1}Ca\textsubscript{1}. The results demonstrate that the 8G5 IgG recognizes a conformation of gelsolin or brevin induced by binding of an easily exchangeable Ca\textsuperscript{2+} ion. Actin is not required for this conformational change, and the antibody discriminates, for example, GCa\textsubscript{3} from G and GCa\textsubscript{1}. A 4F8 IgA-Sepharose column retained brevin or gelsolin in 0.1 mM CaCl\textsubscript{2}-containing buffers, but, like the 8G5 IgG, released these molecules when eluted with 4 mM EGTA. The 4F8 IgA column also retained gelsolin or brevin-actin-Ca\textsuperscript{2+} complexes, for example, as BA\textsubscript{0}Ca\textsubscript{2} or higher oligomers, in 0.1 mM CaCl\textsubscript{2}. No protein was recovered, however, upon elution with 4 mM EGTA, but elution with 0.1 M glycine-HCl, pH 2.8, released bound brevin or gelsolin and actin. Similarly, preformed brevin-actin-Ca\textsuperscript{2+} complex, equilibrated with EGTA, was retained by 4F8 IgA-Sepharose. The results demonstrate that the 4F8 IgA recognizes a conformation of gelsolin or brevin that is maintained and presumably induced by binding of a nonexchangeable Ca\textsuperscript{2+} ion that is trapped in the complex.

G Gelsolin (G) is a 90,000-mol-wt Ca\textsuperscript{2+}-binding, actin-associated protein that is found in a variety of tissues (18, 21). Brevin (B) is a plasma protein closely related to gelsolin, with a molecular weight of 92,000 (6, 9, 10, 16) that has been shown to have an additional sequence of 25 amino acids at its amino-terminal end (22, 24). Gelsolin and brevin are reported to sever actin filaments (6, 14, 16, 25) and both molecules show a Ca\textsuperscript{2+} dependence for this activity. Gelsolin has been shown to have two Ca\textsuperscript{2+}-binding sites each with a K\textsubscript{D} of ~1 \mu M (5, 25). Both gelsolin and brevin will form complexes with G-actin (A) (5, 7, 11, 23). Using nitrobenzoxadiazol-actin, we have established that gelsolin has two actin-binding sites (5). These can be distinguished by the stability of the complexes they form with actin. GA\textsubscript{1}Ca\textsubscript{1} is extraordinarily stable even in the presence of EGTA and can be isolated by a variety of methods (2, 13, 14). While GA\textsubscript{2}Ca\textsubscript{2} can be isolated by gel filtration or sucrose gradient sedimentation (13), it requires, in contrast to GA\textsubscript{1}Ca\textsubscript{1}, the presence of micromolar Ca\textsuperscript{2+} concentrations for stability. We refer to the nonexchangeable or slowly exchanging actin and Ca\textsuperscript{2+}-binding sites that are occupied in the EGTA-stable complex as actin site I and calcium site I, respectively, and will refer to the more rapidly exchanging sites as actin site II and calcium site II, respectively. The complexes will cap the preferred end of actin filaments and slow dilution-induced depolymerization (3) or serve as nuclei when added to G-actin solutions under polymerizing conditions (14, 20, 23). The capping/nucleation function appears to be less Ca\textsuperscript{2+} sensitive (14, 26) than the severing function. Brevin has also been shown to cap and nucleate actin filaments (7). We have shown that the complexes will cap but not cut actin filaments and that severing only occurs if site I is unoccupied when gelsolin is mixed with F-actin (3).

There is relatively little evidence for ligand-induced confor-
mational changes in gelsolin or brevin. We have studied the hydrodynamic behavior of platelet gelsolin and gelsolin-actin complexes (13) and neither show appreciable changes in their Stokes radius or sedimentation value in the presence of EGTA or 100 μM CaCl₂. Gelsolin, for example, has a Stokes radius of 4.15 nm and an S value of 5.2 in either case. This is in marked contrast to the large conformational change which accompanies the binding of Ca²⁺ to villin, an actin-severing protein isolated from microvilli (12). This large conformational change is associated with Ca²⁺ binding to an 8,700-mol-wt fragment, the “head piece,” and does not occur when Ca²⁺ binds to villin core (12). We now report the identification of two murine monoclonal antibodies, 8G5 IgG and 4F8 IgA, that recognize conformational differences between the Ca²⁺-liganded and -unliganded forms of gelsolin and brevin. One of these antibodies, 8G5 IgG, detects changes in both gelsolin and brevin that appear to occur when calcium site II is occupied in GCa2 or BCa2. The other, 4F8 IgA, detects changes in brevin and gelsolin that appear to be associated with binding a Ca²⁺ ion at site I to form BCa1 or GCa1. These conformational changes do not require the presence of actin.

**Materials and Methods**

**Preparation of Hybridomas**

Male BALB/c mice received a primary immunization with ~20 μg of purified human gelsolin-actin complex and were subsequently immunized with 10 μg of the immunogen in 2-wk intervals over a period of ~3 mo. When high titer serum immunoglobulin was detected, the animals were boosted with 10 μg of immunogen on 3 consecutive d, and then sacrificed. A cell suspension was prepared in Dulbecco’s modified Eagle’s medium (DME) and 200 x 10⁶ spleen cells were mixed with 67 x 10⁶ A653 mouse myeloma cells then pelleted by centrifugation at 200 g for 5 rain. Fusion was induced by resuspending the cell pellet in 40% polyethylene glycol in DME and incubating at 37°C for 4 h. The 8G5 or 4F8 hybridomas were grown in CEM serum-free media supplemented with transferrin, insulin, and selenium (Scott Laboratories, Inc., Fiskeville, RI) at an initial density of 2.5 x 10⁶ cells per cm² in T-175 flasks (Falcon Labware). After 3 d, the medium was removed and separated from the cells by low speed centrifugation at 200 g for 5 min. The cells were then resuspended in fresh medium and seeded into T-175 flasks. Approximately 5 liters of serum-free medium were processed to obtain purified antibody.

**ELISA**

ELISAs were used to identify hybridomas secreting antibody against gelsolin. Briefly, 0.5 μg of partially purified gelsolin-actin complex was absorbed to 96-well microtiter plates pre- treated with mouse peritoneal macrophages. 24 h later an equal volume of DME containing 10% fetal calf serum and 2 x 10⁻⁴ M hypoxanthine, 3.2 x 10⁻⁴ M thymidine, and 8 x 10⁻⁴ M methotrexate was added to each well to select for hybridomas. After 2 wk of growth, the plates were screened by an enzyme-linked immunosorbent assay (ELISA) and the cells from positive wells were expanded to 24-well plates. These wells were screened a second time by immunoblotting (19) on whole platelet extracts fractionated on polyacrylamide gels (15). The wells that were positive on the ELISA and immunoblots were cloned by single-cell deposition onto thymocyte-seeded 96-well microtiter plates using an Ortho Cytoflurograph 50H (Ortho Instruments, Westwood, MA). The clones were tested for antibody production by ELISA, immunoblot, and indirect immunofluorescence.

**Immunoblotting**

Routine immunoblotting was done following the methods of Towbin et al. (19). In some experiments we have tested the effects of EGTA on the binding of individual monoclonal antibodies. In these experiments, proteins were separated by SDS gel electrophoresis and transferred electrophoretically to nitrocellulose filters (Type GF/B, 22 cm, Millipore/Continental Water Systems, Bedford, MA) in 0.192 M glycine, 0.025 M Tris, 20% methanol. The nitrocellulose sheets were then blocked in 3% bovine serum albumin plus 5% calf serum in PBS with 0.1% NaN₃ for 12–18 h. The sheets were divided and one-half were washed 3 times with Trition X-100–PBS plus 5 mM EGTA while the other half were washed with PBS plus 0.05% Trition X-100 plus 0.1 mM CaCl₂. The sheets were then incubated for 2 h at 37°C with harvest fluids with and without 8 mM EGTA. To remove unbound antibody the sheets were washed three times in PBS plus 0.05% Trition X-100 either with Ca²⁺ or without EGTA, but without NaN₃. A fourth wash was done in PBS plus 0.05% Trition X-100 and Ca²⁺ to remove all the nitrocellulose sheets to the same buffer. The second step antibody was added and incubated at 37°C for 2 h. Finally, the sheets were washed three times with PBS plus 0.05% Trition X-100 without NaN₃ then incubated with substrate, 4-chloro-l-naphthol (Sigma Chemical Co., St. Louis, MO). The reaction was stopped after 5–30 min by washing with distilled water.

**Cross-reactivity of Anti-gelsolin Antibodies**

To prepare extracts from different types of cultured cells, ~1–2 x 10⁶ cells of each type were washed with PBS, resuspended into hot sample buffer, and heated at 90°C for 5 min. These whole cell extracts, along with platelet extract prepared as described previously (2, 14) and partially purified gelsolin-actin complex were run on 7.5–15% SDS polyacrylamide gradient gels and either stained with Coomassie Blue or blotted with antibody-containing harvest fluid.

**Antigen Digestion Experiments**

Partially purified gelsolin-actin complex was digested with trypsin at molar ratios of 100:1 and 1:0.01. Time points were taken at 0, 10, 30, 90, and 240 min by addition of hot SDS electrophoresis sample buffer followed by heating for 3 min at 100°C. Alternatively dithiothreitol was added to 2 mM to stop the reaction before SDS addition. Samples were run on 15% polyacrylamide gels (15) and were either stained with Coomassie Blue or were electrophoretically transferred to nitrocellulose, and then blotted with antibody-containing harvest fluid.

**Growth in Serum-free Medium**

The 8G5 or 4F8 hybridomas were grown in CEM serum-free media supplemented with transferrin, insulin, and selenium (Scott Laboratories, Inc., Fiskeville, RI) at an initial density of 2.5 x 10⁶ cells per cm² in T-175 flasks (Falcon Labware). After 3 d, the medium was removed and separated from the cells by low speed centrifugation at 200 g for 5 min. The cells were then resuspended in fresh medium and seeded into T-175 flasks. Approximately 5 liters of serum-free medium were processed to obtain purified antibody.

**Concentration of Serum-free Medium and Purification of Antibodies**

Serum-free harvest fluid was concentrated ~15-fold using a Millipore Miniant System (Millipore/Continental Water Systems). The IgG in the concentrated from 8G5 was purified on Protein A-agarose (Sigma Chemical Co., St. Louis, MO). The recovery was ~110 mg of 8G5 IgG per 5 liters of serum-free medium determined using an extinction coefficient of 1.4 at OD₂₈₀ for a concentration of 1 mg/ml. The IgA in the concentrate from 4F8 harvest fluid was purified by (NH₄)₂SO₄ precipitation and chromatography on G-150 Superfine. The final yield was ~40–50 mg per 5 liters of serum-free medium.

**Coupling of Antibody to Cyanogen Bromide-activated Agarose**

Purified 8G5 IgG or 4F8 IgA was dialyzed exhaustively against 0.5 M NaCl, 0.1 M NaHCO₃, pH 8.8, then coupled to CNBr-activated Sepharose 4B (Sigma Chemical Co.) following the procedures outlined by Pharmacia (Pharmacia Fine Chemicals, Piscataway, NJ). Briefly, the CNBr-Sepharose was hydrated by washing five times in 1 M HCl. The final gel pellet was washed rapidly with 0.5 M NaCl, 0.1 M NaHCO₃, pH 8.8, and then resuspended in approximately an equal volume of 8G5 antibody solution at 9 mg/ml in 0.5 M NaCl, 0.1 M NaHCO₃, and allowed to react overnight at 4°C with gentle agitation. The supernatant was removed by centrifugation at 2,500 g for 2 min and the
Miscellaneous were done following Towbin et al. (19). Protein concentrations were determined on SIDS polyacrylamide gels were run according to Laemmli (15). Immunoblots for brevin isolation.

Platelet Preparation

Human platelets were washed and high salt extracts were prepared as described previously (13, 14), modified as indicated by Bryan (2). The high salt extracts were dialyzed against 40 mM KCl, 0.1 mM EGTA, 10 mM imidazole, pH 7.2, to produce a contractile gel consisting principally of actin, myosin, actin-binding protein, alpha-actinin, and tropomyosin. Some gelsolin was lost at this step, presumably by binding to filament ends. For isolation of gelsolin–actin complexes, G\textsubscript{A}C\textsubscript{A}, and G\textsubscript{A}C\textsubscript{C}, the contractile material was removed by centrifugation at 100,000 g for 60 min and the resulting supernatant was dialyzed against 40 mM KCl, 0.1 mM CaCl\textsubscript{2}, 1.0 mM Na\textsubscript{2}SO\textsubscript{4}, and 10 mM imidazole, pH 7.2. Alternatively, the supernatant was dialyzed against 6 M urea, 0.1 mM EGTA, 1.0 mM Na\textsubscript{2}SO\textsubscript{4}, and 10 mM imidazole, pH 7.2, to dissociate gelsolin–actin complexes. The urea was then removed by dialysis against 40 mM KCl, 0.1 mM CaCl\textsubscript{2}, 1.0 mM Na\textsubscript{2}SO\textsubscript{4}, 10 mM imidazole, pH 7.2. We have shown previously that, under these conditions, gelsolin either is not denatured or renatures easily (5).

Preparation of Serum for Brevin Isolation

The citrated plasma recovered after platelet isolation was used to prepare human brevin. The plasma was centrifuged at 20,000 g for 30 min. The supernatant was then dialyzed against 140 mM NaCl, 3 mM CaCl\textsubscript{2}, 1.0 mM Na\textsubscript{2}SO\textsubscript{4}, 10 mM imidazole, pH 7.2, to induce clot formation. The dialysis was done at room temperature with three changes of buffer using 4 liters of buffer per liter of plasma per change. The fibrin clot was removed by centrifugation at 20,000 g for 20 min. This serum was rapidly frozen in 50-ml aliquots in liquid nitrogen and stored at -70°C. Before use, 50–100 ml were thawed and centrifuged at 100,000 g for 60 min. The supernatant was used for brevin isolation.

Miscellaneous

SDS polyacrylamide gels were run according to Laemmli (15). Immunoblots were done following Towbin et al. (19). Protein concentrations were determined by a modification of the Bradford method (1) using bovine serum albumin as a standard.

Results

Characterization of Anti-Gelsolins

We have characterized antigen–antibody binding using several procedures including (a) immunoblotting of various cell extracts, (b) immunoblotting of intact and proteolytically fragmented gelsolin, (c) immunoblotting of gelsolin either in the presence of Ca\textsuperscript{2+} or EGTA, and (d) by analyzing the material retained by antibody coupled to Sepharose. We have identified the antibodies produced by 8G5 as an IgG and by 4F8 as an IgA using antibody typing reagents and by comparison of the molecular weights of their heavy and light chains with known standards by SDS PAGE (data not shown).

The results of the blotting experiments for the various cell extracts and for intact and fragmented gelsolin are shown in Figs. 1 and 2. Immunoblots of complex mixtures of protein from human platelets, WI-38 cells, or human foreskin fibroblasts gave a single reactive component of 90,000-mol-wt that migrated with purified gelsolin standards (Fig. 1). The two nonhuman cell types, 3T3 and NRK/RSV cells, did not cross-react at the protein concentrations used here. The immunoblots of the partial tryptic digest, Fig. 2, suggest that the epitope for 8G5 IgG is located on a peptide near one end of gelsolin since cleavage to a 70,000-mol-wt fragment greatly reduced cross-reactivity. 4F8 IgA appears to recognize an epitope deeper in the molecule since it cross-reacts with both the 70,000-mol-wt peptide and a 50,000-mol-wt peptide. In additional cleavage experiments with other proteases, we have shown that both the 4F8 and 8G5 epitopes are on the C terminal half of brevin (4).

Note that there was no evidence for a cross-reaction with actin in any of the immunoblotting experiments. In the platelet extracts we estimate that the relative concentration of

Figure 1. Immunoblots of cell and platelet extracts and partially purified gelsolin–actin complex. Coomassie Blue–stained gels, on the left, are shown paired with corresponding immunoblots, on the right. 8G5 harvest fluid was used unless otherwise indicated. Lane A, molecular weight markers: 94,000, phosphorylase B; bovine serum albumin, 68,000; ovalbumin, 43,000; carbonic anhydrase, 30,000; soybean trypsin inhibitor, 21,000; lysozyme, 14,300. Lanes B and C, Swiss 3T3 cells. Lanes D and E, RSV/NRK cells. Lanes F and G, human foreskin fibroblasts. Lanes H and I, WI-38 cells. Lane J, platelet extract. Lanes K and L, immunoblots with 4F8 (K) and 8G5 (L) harvest fluids. No reactivity was detected with NRK/RSV and 3T3 cells whereas a single band at 90,000-mol-wt was stained in each of the other samples.
Figure 2. Characterization of anti-gelsolin antibodies. Partially purified gelsolin–actin complex (1 mg/ml) was digested with trypsin at molar ratios of 100:1 (lanes 1–5) and 1,000:1 (lanes 6–9). Time points were taken at 0 (lane 1), 10, 30, 90, and 240 (lanes 2–5 and 6–9) min; proteolysis was stopped by addition of SDS PAGE sample buffer. Four identical 15% gels were run: a Coomassie Blue-stained gel is shown in A. The molecular weight standards are the same as those shown in Fig. 1. Their positions are marked with small arrowheads on the left side. After electrophoretic transfer to nitrocellulose, immunoblots were done using the three monoclonal anti-gelsolin antibodies. B shows the fragment staining pattern using 8G5 IgG and C shows the staining pattern using 4F8 IgA.

The Antibodies Recognize Ca²⁺-induced Conformations

Fig. 3 demonstrates the specificity of the 8G5 and 4F8 antibodies for specific Ca²⁺-induced gelsolin conformations. Various concentrations of a partially degraded gelsolin preparation were run in duplicate on SDS polyacrylamide gels then actin must be 50–100 times greater than gelsolin, yet the actin band was never stained. Similarly, we have done immunoblots on rabbit skeletal muscle actin and detected no cross-reactivity with actin bands of 5–15 µg and purified 8G5 IgG concentrations of 10–50 µg/ml. Under the same blotting conditions, however, we could detect 1–2 µg of human gelsolin using <0.1 µg/ml of purified 8G5 IgG.
transferred to nitrocellulose filters. The filters were cut in half and incubated with either 8G5 or 4F8 antibodies in either Ca²⁺ or 1 mM EGTA-containing solutions. The results show that both antibodies have different affinities for gelsolin depending upon the presence of Ca²⁺ or EGTA, and gave a strong positive signal if Ca²⁺ was present but showed little recognition in the absence of Ca²⁺. In the presence of Ca²⁺, 8G5 IgG reacted strongly with a single band at 90,000-mol-wt, but gave only a barely detectable signal with ~2 µg of human gelsolin if EGTA was present during the antibody-binding step. 4F8 IgA, under the same conditions, reacted strongly with the 90,000-mol-wt species and with fragments at 63,000, 53,000, and 47,000-49,000-mol-wt. A murine polyclonal antibody, used here as a control, did not show any particular sensitivity to Ca²⁺ levels although there is some attenuation of the signal on immunoblots (Fig. 3).

Note that although we have not directly demonstrated Ca²⁺ binding to gelsolin after electrophoresis in SDS, we have shown that gelsolin will bind ¹²⁵I-actin in an overlay procedure (17) and that this binding is strongly promoted by added Ca²⁺ (18).

**Purification of Gelsolin-Actin Complexes Using 8G5 IgG**

The affinity of 8G5 IgG for a Ca²⁺-induced conformation of gelsolin provides a facile method for purification of gelsolin and brevin. The results of one gelsolin purification done at higher ionic strength, 300 mM KCl, are shown in Fig. 4. An 8G5 IgG-Sepharose column, equilibrated with 300 mM KCl, 0.1 mM CaCl₂, 1.0 mM NaN₃, and 10 mM imidazole, pH 7.2, was loaded with a gelsolin-containing platelet extract adjusted to 0.1 mM Ca²⁺. The same buffer was used to wash the column after loading until the concentration of protein in the eluate was <5 µg/ml. The protein eluted with 300 mM KCl, 4 mM EGTA, 1.0 mM NaN₃, and 10 mM imidazole at pH 7.2. The protein concentration of the initial
Figure 4. Immunoaffinity purification of the gelsolin–actin complex. A platelet cytosol preparation containing Ca\(^{2+}\) was applied to the 8G5 IgG-Sepharose column. The preparation of the extract and the 8G5 IgG-Sepharose are described in Materials and Methods. Briefly, 25 ml of extract at ~10 mg/ml was diluted to 3.0 mg/ml at a final KCl concentration of 0.3 M and a final Ca\(^{2+}\) concentration of 0.1 mM then loaded on the column. The top panel shows the Coomassie Blue-stained gel of selected fractions. The lower panel shows the protein profile. In this run, the buffer was switched to 0.3 M KCl + 4 mM EGTA at the arrow. The fraction size was 10 ml from fraction 1 to 22 and 1.0 ml from fraction 23 on. Note that the actin that is not complexed with gelsolin is not retained by the antibody.

Extract was 3–4 mg/ml and we routinely recover 20–40 \(\mu\)g of gelsolin per ml of this extract. This can be used to provide a minimum estimate for the concentration of gelsolin in platelet extracts, but does not account for gelsolin that sediments with the contractile gel or that may sediment when the particulate fraction is removed. The average recovery from four experiments was 0.65 mg of gelsolin per 100 mg of protein in the original high salt extract. The ratio of actin to gelsolin in the fractions eluted with EGTA is greater than one but less than two, indicating that a mixture of GA\(_1\) and GA\(_2\) and perhaps higher oligomers bound to the antibody. We have established previously that GA\(_1\)Ca\(_i\) is stable in EGTA (14) and have chromatographed the fraction eluted from 8G5 IgG-Sepharose on Agarose A5m to establish that the eluted species is GA\(_1\)Ca\(_i\). The results (not shown) indicate that a 130,000-mol-wt species containing actin and gelsolin is present. Finally, we
have prepared GA, Ca, loaded with $^{45}$Ca$^{2+}$, as described by Kurth and Bryan (13). This was loaded on 8G5 IgG-Sepharose after addition of Ca$^{2+}$ to 0.1 mM. The $^{45}$Ca$^{2+}$ eluted after EGTA addition and was complexed with GA, as judged by chromatography on agarose A5m. The results strongly indicate that the conformation recognized by the 8G5 IgG is induced or stabilized by the binding of the second Ca$^{2+}$ ion to gelsolin. We note that 8G5 IgG does have low affinity for other unliganded forms of gelsolin since loading a platelet extract dialyzed versus 0.1 or 1 mM EGTA results in the partial retention of gelsolin-actin complexes with elution as a distinct trailing shoulder on the main protein peak. This agrees with the low level of binding of the antibody in EGTA to the highest concentrations of gelsolin in the immunoblots shown in Fig. 3.

Our absolute yield of gelsolin per column run is ~5-6 mg of protein, a value obtained by summing the protein across the gelsolin peak. Loading the column with more extract results in gelsolin appearing in the unbound protein fraction. We calculate, therefore, that only 8 to 10% of the coupled antibody can bind with the antigen. We have not systematically varied the antibody to CNBr-Sepharose ratios to try to optimize for active antibody.

**Purification of Free Gelsolin or Brevin**

To demonstrate the usefulness of 8G5 IgG and also to see if a complexed actin was required for antibody recognition, we have purified both gelsolin and the closely related plasma protein, brevin, directly. To purify gelsolin from platelets, it is necessary to split the gelsolin-actin complexes that form spontaneously (13). We have used dialysis against 6 M urea to break these complexes (13) as described in Materials and Methods. The strategy was to dialyze versus urea and EGTA to weaken the complex and eliminate ATP to maximize the denaturation of actin. The urea was then removed and the Ca$^{2+}$ level raised by dialysis. Chromatography of this material under the same conditions used for isolation of the gelsolin-actin complex yielded free gelsolin. 8G5 IgG-Sepharose did not retain actin unless it was complexed to gelsolin. To test further whether actin was necessary for the recognition of gelsolin by 8G5 IgG, we have used the antibody column in the purification of human plasma brevin. The result is shown in Fig. 5. 80-100 ml of serum prepared as described in Materials and Methods were loaded on 8G5 IgG-Sepharose and eluted with EGTA. In this particular experiment, the serum was loaded in 140 mM NaCl, 3 mM CaCl$_2$, 1.0 mM NaN$_3$, 10 mM imidazole at pH 7.2. The column was then washed with 0.3 M KCl, 0.1 mM CaCl$_2$, 1.0 mM NaN$_3$, and 10 mM imidazole at pH 7.2. Brevin was eluted by substituting 4 mM EGTA for 0.1 mM CaCl$_2$ in the same buffer. The eluted material consisted of brevin and small amounts of human IgG heavy and light chains which appeared to stick either to the mouse IgG or to the agarose even in high salt. Their concentration could be reduced by prolonged washing.

![Figure 5. Immunoaffinity purification of human serum brevin. Citrated human plasma was dialyzed against Ca$^{2+}$-containing buffers to induce clotting as described in Materials and Methods. The serum, 100 ml at 38 mg/ml, was loaded on an 8G5 IgG-Sepharose column. In this experiment, the buffer was switched to 0.3 M KCl plus 0.1 mM CaCl$_2$ at the first arrow and to 0.3 M KCl plus 4 mM EGTA at the second arrow. The fraction size was 10 ml from fraction 1 to 17, 5 ml from fraction 18 to 25, and 1 ml from fraction 26 on. Selected fractions were analyzed by SDS PAGE and are shown in the inset. The fractions, from left to right, are numbers 34, 36, 38, 40, 42, and 44.](image-url)
with the high salt buffer before elution with EGTA. We routinely recover ~50–100 μg of brevin per ml of serum. A similar result was obtained using urea-treated platelet extracts; gelsolin was the major component eluted with EGTA. The absolute yield of brevin, ~5–6 mg, was the same as described above for gelsolin. These results demonstrate that 8G5 IgG recognizes both gelsolin and brevin.

**Activity Assays**

We have tested both the gelsolin, gelsolin–actin complexes, and brevin eluted from 8G5 IgG-Sepharose for their ability to cap or sever filaments. The gelsolin preparations are equivalent to those we have prepared using DNAse I-agarose affinity chromatography (2, 20) or more standard biochemical methods (2, 14). The brevin purified by immunoaffinity chromatography is active both in severing and nucleation assays (data not shown).

**Comparison of 8G5 IgG and 4F8 IgA-Sepharose**

We have used 4F8 IgA-Sepharose to purify brevin from serum with results identical to those described in Fig. 5 for 8G5 IgG-Sepharose. Brevin was retained in 0.1 mM Ca\(^{2+}\)-containing buffers and released by 4 mM EGTA-containing buffers. We have also prepared brevin–actin–Ca\(^{2+}\) complexes by mixing purified brevin with a 2.5-fold molar excess of actin in 0.2 mM CaCl\(_2\), 50 mM NaCl, 2 mM Tris-HCl, pH 8, and 0.5 mM ATP. Chromatography of this material on 4F8 IgA-Sepharose, shown in Fig. 6, gave markedly different results. The complex, BA\(_2iCa\), was retained even in 10 mM EGTA and 0.6 M NaCl, but was released by elution with 0.1 M glycine-HCl, pH 2.8. Finally, we have equilibrated the complex with 1 mM EGTA and loaded this on 4F8 IgA-Sepharose equilibrated with buffers containing 1 mM EGTA. The complex was retained, but could be eluted at pH 2.8 as shown in Fig. 7.

A summary of the binding behavior of the various forms of brevin and gelsolin–actin complexes on the antibody columns is given in Table I. We have tested all the possible combinations and find that binding of all species with 8G5 requires Ca\(^{2+}\), while 4F8 retains brevin–actin and gelsolin–actin complexes in Ca\(^{2+}\) or EGTA.

**Discussion**

This report demonstrates the usefulness of immunopurification for gelsolin and brevin. We can obtain milligram quantities of either protein in 1 or 2 d. In addition, the results demonstrate the specificity of two monoclonal antibodies, an IgG and an IgA, for Ca\(^{2+}\)-induced conformations in the gelsolin molecule. The initial immunogen was the actin–gelsolin complex, GA\(_2iCa\), but neither of the antibodies recognizes actin on immunoblots. In addition, neither 4F8 IgA nor 8G5 IgG eliminated the actin–gelsolin complex by immunoaffinity chromatography.
Figure 7. Chromatography of brevin–actin complex on 4F8 IgG-Sepharose in EGTA. Approximately 1.0 mg of brevin–actin complex was dialyzed exhaustively against 0.3 M NaCl, 1 mM EGTA, 2 mM NaN₃, and 10 mM imidazole, pH 7.2, then loaded on the 4F8 IgA-Sepharose column described in Fig. 6 equilibrated with the EGTA-containing dialysis buffer. The material that is not retained is mainly excess actin. Elution with 0.1 M glycine-HCl, pH 2.8, at the arrow, releases the brevin and actin. Preliminary characterization indicates that low pH dissociates the complex, presumably by denaturing the actin. The brevin does retain severing activity. The fraction size was 0.8 ml. Selected fractions were analyzed by SDS PAGE and are shown in the upper panel. The first lane shows the profile for the starting material.

nor 8G5 IgG-Sepharose retains actin unless it is associated with gelsolin. Using 4F8 IgA and 8G5 IgG-Sepharose we have characterized the antigen–antibody reactions. At free Ca²⁺ concentrations that will saturate the Ca²⁺-binding sites of gelsolin, whose $K_D$'s are ~1 μM, the antibodies retain $GA_2Ca_2$ and possibly higher oligomers containing additional actin monomers from extracts prepared from activated platelets. The addition of EGTA, which will remove one bound Ca²⁺ from $GA_2Ca_2$, but not the other (13), releases the retained gelsolin and actin from 8G5 IgG, but not 4F8 IgA-Sepharose.

The material released from 8G5 IgG-Sepharose chromatographs on gel-filtration as a 130,000-mol-wt species with the characteristics of $GA_2Ca_2$, (13). Our interpretation is that 8G5 IgG recognizes $GA_2Ca_2$ with high affinity, but has a markedly reduced affinity for $GA_1Ca_1$. We have done similar experiments with reconstituted brevin–actin complexes with the same results. 4F8 IgA, on the other hand, recognizes both $GA_1Ca_1$ and $BA_1Ca_1$ with high affinity and will retain these complexes in the presence of EGTA.

The behavior of gelsolin is the same as the gelsolin–actin
complexes on 8G5 IgG-Sepharose and we infer that this antibody binds GCA2, more tightly than G or GCA1, and that actin is not important for these interactions. The same conclusions hold for brevin. This altered affinity must reflect a conformational change in gelsolin or brevin that occurs when a Ca2+ ion binds at calcium site II, the more easily exchangeable site. The conformational change appears to be small since we have not detected changes in the Stokes radius or sedimentation value of gelsolin in the presence of Ca2+ or EGTA (13). On the other hand there is a marked difference in the binding affinity at actin site II between GACa2 and GACTa2 (5). We have estimated the Kd for binding of the second actin to be 30 nM for GACa2 and <0.1 nM for GACTa2.

4F8 IgA must recognize a second conformation since GACa2 and BACa2 are retained by 4F8 IgA-Sepharose. Actin does not appear to be required for the conformational change recognized by 4F8 IgA since brevin, as BCa2, is retained by the column. However, as we have shown previously (13), the gelsolin-actin-Ca2+ complex, GACa2, traps a Ca2+ ion and is stable in EGTA. We have shown that BACa2 also traps a Ca2+ ion (J. Bryan, unpublished data). The present results show that an actin bound at actin site II can stabilize the brevin or gelsolin conformation recognized by 4F8 IgA and prevent elution with EGTA. We infer that this conformation is induced by binding of a Ca2+ ion at calcium site I, where it is then trapped in the complex.

Finally, we cannot pinpoint where the physical location of the conformational change recognized by 8G5 IgG is within the protein using the immunoblots of the proteolyzed fragments shown in Fig. 2. The data suggest that the removal of a 20,000-mol-wt piece destroys 8G5 IgG recognition. We have been unable to recover this 20,000-mol-wt fragment and demonstrate Ca2+-sensitive antibody binding. 4F8 IgA recognizes a separate epitope that is present on several proteolytic fragments with molecular weights of 63,000, 53,000, and 47,000–49,000. Each of these fragments appears to retain a calcium-binding site since Ca2+ is required for antibody recognition. Our preliminary results with other proteases indicate that both epitopes are on the C terminal half of the molecule (4). The NH2-terminal half of the molecule has the actin-binding domains and retains all of the filament severing activity. Proteolysis separates the Ca2+- and actin-binding domains and produces fragments that sever but are no longer regulated by Ca2+. We are currently working to understand the interactions between these domains on gelsolin and brevin molecules.