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Profilin Forms Tetramers That Bind to G-Actin

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Profilin binds to G-actin and affects polymerization. However, regulation of profilin function is generally unknown and controversy exists regarding profilin effects on actin polymerization. Because protein-protein interactions are implicated in many cellular responses, human platelet profilin self-association and actin interaction was examined. Silver stained SDS-PAGE of poly-l-proline/sepharose 4B column purified profilin revealed the presence of profilin (14.8 kD) and extraneous higher bands (primarily 30 kD and 58.5 kD). Re-electrophoretic analysis of gel electroelution purified profilin yielded predominantly 14.8 kD and 58.5 kD proteins. Rabbit IgG antibodies made against gel electroelution-purified profilin recognized all profilin sizes on immunoblots. Capillary electrophoresis of profilin in solution produced a single peak that resolved into three distinct peaks upon addition of reducing agent or high salt conditions. Further, G-actin did not bind to 14.8 kD profilin on immunoblot overlay assays, but surprisingly bound only to 58.5 kD profilin. The data indicate that monomeric profilin forms tetramers which are the relevant high affinity actin-binding form.

Profilins are ubiquitous cytoskeletal proteins (1–5) that affect actin polymerization and signal-transduction (6,7). The ability of profilin to sequester G-actin monomers is considered an underlying mechanism whereby actin polymerization is prevented. However, kinetic studies on the interaction between profilin and actin in a reconstituted system (8) do not support the existence of a high affinity profilin-actin complex, as suggested from initial work to isolate and crystalize profilactin (1,9). Furthermore, recent evidence indicates that profilin may actually enhance actin polymerization by promoting the exchange of ADP-with ATP-bound to actin monomers (4,10–12). Profilin may also lower the critical concentration for actin polymerization by dissociating actin from thymosin β4, another major G-actin sequestering molecule (11). In support, profilin and profilin-actin complexes microinjected into rat kidney cells (13) led to decreased and increased F-actin, respectively. The dual nature of profilin effects on actin may suggest multiple profilin states or regulatory mechanisms exist.

Protein-protein associations often occur to promote cellular responses, though few examples of homologous multimerization-dependent regulation of protein function exist. Established methods of profilin isolation have often yielded extraneous and unidentified proteins (e.g., 1,2,14–19) that are $\geq$2 times the recognized size of the 12–15 kD cytoskeletal molecule. Considering the varied and contrasting effects observed for profilin, and the larger proteins associated with profilin isolation procedures, multimerization was examined.

METHODS

Reagents

CNBr-activated sepharose 4B was purchased from Pharmacia (Piscataway, NJ) and poly(l-proline) (PLP; 10,000–30,000 MW) was purchased from Sigma Chemical Co. (St. Louis, MO). Silver staining kits were purchased from either Pierce

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Chemical Co. (Rockford, IL) or BioRad (Hercules, CA) and supported nitrocellulose transfer membranes were from Research Products International Corp. (Mount Prospect, IL). The antibodies were mouse monoclonal IgG anti-chicken actin (c4 clone; ICN, Irvine, CA), goat anti-mouse IgG-HRP and anti-rabbit IgG-HRP conjugated (Pierce Chemical Co.). G-actin was supplied by Sigma Chemical Co. for some experiments or otherwise purified from human platelets as described below.

Profilin and Actin Isolation

(a) Poly(l-proline)-sepharose 4B affinity chromatography. Profilin and actin were isolated essentially as described (8,17,20,21). Human platelets (isolated within 7–10 days) were washed (135 mM NaCl, 15 mM trisodium citrate, 11.1 mM glucose, 10 mM Tris-HCl, pH 6.5) and centrifuged (1,000g × 30 m, 22°C) to yield a pellet that was suspended in 5 vol ice-cold lyzate buffer (0.1 mM ATP, 0.5 mM DTT, 1% Triton X-100, 1% DMSO, 10–20 μg/ml each of leupeptin, aprotinin and pepstatin, 5 mM Tris-HCl, pH 7.2) and sonicated (continuous output control setting 2 × 60 s, Sonifier cell disruptor, Branson Sonic Power Co.). The lyzate was centrifuged (12,000g × 35 m, 4°C) and supernatant from the equivalent of 10 ml packed platelets was poured onto a 10 ml poly(l-proline)-sepharose 4B column. Actin and profilin eluted with 4M and 8M urea, respectively, were concentrated by centrifugation (centriprep-3, Amicon Inc., Beverly, MA). Profilin was initially washed in G-buffer (0.1 mM $CaCl_2$, 0.2 mM ATP, 0.5 mM DTT, 2 mM Tris-HCl, pH 7.2), concentrated (1–3 mg/ml) and stored in 2 mM Tris-HCl (pH 7.4)/0.1 mM $CaCl_2$ at −20°C until use and actin was stored in G-buffer.

(b) SDS-polyacrylamide gel electrophoresis and electroelution. Samples of profilin and actin were solubilized in standard sample buffer (unless otherwise noted) and electrophoresed by 15% SDS-PAGE. Protein bands were identified by either Coomassie blue or silver stain techniques. In some instances, gel slices containing profilin (14.8 kD) or actin (43 kD) identified by rapid, reversible stain (Diversified Biotech, Boston, MA) were excised and electroeluted into ultrafiltration devices (centricon-3, Amicon Inc.). Following centrifugation (2,900g × 2 hrs) to elute off the running buffer (5mM Tris-HCl, 38.4 mM glycine, 0.02% SDS) the proteins were extensively washed and refiltered with 2 mM Tris-HCl (pH 7.4)/0.1 mM $CaCl_2$ (profilin) or similar buffer containing 0.2 mM adenosine triphosphate and 0.5 mM DTT (actin). Other buffers used for capillary zone electrophoresis of profilin are described below. Profilin isolation was initially verified on immunoblots with rabbit serum anti-human platelet profilin antibodies (herein termed anti-profilin$_1$) kindly provided by Dr. Pascal J. Goldschmidt-Clermont (Johns Hopkins University, Baltimore, MD). Protein determinations were made by BCA assay (Pierce Chemical Co., Rockford, IL) using BSA as the standard.

Anti-profilin Antibody Development

Electroelution purified profilin (above) was conjugated with adjuvant (RIBI Immunochemical Research, Inc., Hamilton, MT) and injected into ten discrete locations (per RIBI protocol) into New Zealand White rabbits. Rabbit serum anti-profilin IgG was purified by thiophilic adsorption chromatography (Pierce Chemical Co., Rockford, IL) to yield an average peak fraction concentration of 4.5 mg IgG/ml. Anti-profilin IgG antibodies were screened on Western immunoblots containing antigen.

Western Immunoblotting

Proteins identified by SDS-PAGE were transferred onto supported nitrocellulose (semi-dry graphite electroblotter; Millipore, Bedford, MA) and blocked with a solution of 4% non-fat powdered milk, 0.1% BSA, and 0.02% NaN_3 in TBS (pH 7.4) for >1 hr. Membranes were subsequently washed with TBS, incubated for 2 hr with primary antibody (1:500), washed in TBS and exposed to appropriate secondary HRP-conjugated antibody for protein visualization with 4-chloronapthal. The exposure times of immunoblots with 4-chloronapthal varied in order to maximize identification. In some experiments the immunoblots containing electroelution purified profilin were incubated with G-actin (3μM in G-buffer × 60 m) before exposure to antibodies (i.e., overlay assay; 22).

All gels and immunoblots shown represent the results of at least three experiments from different human platelet preparations.

Capillary Zone Electrophoresis

Electroelution purified profilin was reconstituted with either G-buffer, HEPES pH 7.3/0.1 mM $CaCl_2$, or cytosolic buffer (in mM: KCl 3, NaCl 20, EGTA 3, $MgSO_4$ 5, NaH_2PO_4 1, Na_2HCO_3 25, $CaCl_2$ 1, HEPES 20, pH = 7.4). Profilin ($\sim$0.2μM) was analyzed by CZE (Waters Quanta 4000, 50μm × 65cm Supelco P150 hydrophilic column, 25°C, 25 kV, detection = 214 nm).

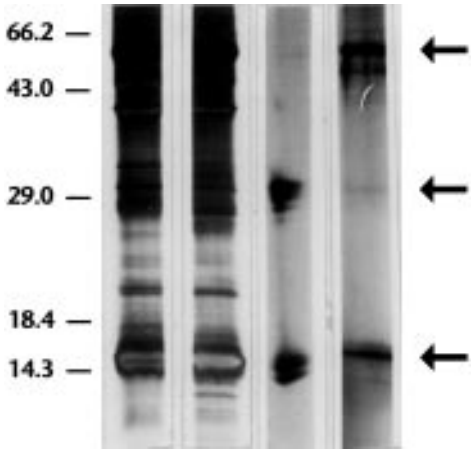

FIG. 1. SDS-PAGE of purified human platelet profilin. *Lane 1* = silver stained gel of poly(l-proline)-sepharose 4B column purified profilin (20 μg) run through 15% SDS-PAGE in sample buffer without reducing agents; *lane 2* = same as lane 1, but in sample buffer containing DTT (0.5 mM), βME (10%), and iodoacetamide (5 mM); *lane 3* = gel slice containing the 14.8 kD protein (which originally migrated as shown in lane 2) was electrophoresed a second time; *lane 4* = initial 14.8 kD protein was electroeluted and rerun (5 μg) on SDS-PAGE. M_r obtained from standards are indicated on the left. Profilin proteins (arrows on the right) indicate calculated M_rs corresponding to 58.5, 30, and 14.8 kD.

RESULTS

Profilin extracted from human platelets by poly(l-proline) sepharose 4B affinity chromatography was initially examined by SDS-PAGE separation. Although this method generally yields highly ($\sim$95%) purified profilin by scanning Coomassie Blue-stained gels (17; and confirmed in our lab), the remaining extraneous proteins become more evident with intentionally overloaded and over-developed silver stained gels (Fig. 1, lanes 1 and 2). Re-electrophoresis of a gel slice that contained 14.8 kD protein, corresponding to the estimated size of known profilins, led to three visible proteins, including major 14.8 kD and 30 kD bands (Fig. 1, lane 3). Electrophoresis of a product electroeluted from the initial 14.8 kD profilin gel slice yielded predominantly 14.8 kD and 58.5 kD proteins (Fig. 1, lane 4). The results indicate higher MW proteins arise from profilin self-association.

Profilin multimerization was verified by immunoblot analysis of samples run in parallel with the SDS-PAGE experiments described above. Anti-profilin antibodies bound to three proteins (14.8, 30 and 58.5 kD) from affinity column-purified profilin (Fig. 2, lane 2) and also recognized electroelution-purified profilin (Fig. 2, lane 3). The order of detection was generally 58.5 kD $\geq$ 14.8 kD $\gg$ 30, but some variability was noted among the relative amounts of each protein from different platelet preparations (e.g., Fig 2, lane 4). The results indicate that tetramers (profilin$_4$) and dimers (profilin$_2$) are formed from monomeric profilin (profilin$_1$). Though it is unclear why profilin identified by immunoblotting from other laboratories appears as a single 12–15 kD species, this may reflect differences in either protein isolation and subsequent antigenic use, or antibody purification and screening techniques that select antibodies directed against profilin$_1$.

Electroelution-purified profilin multimerization in solution was examined by CZE. A single peak was detected for profilin (Fig. 3A) that separated into three peaks upon addition of reducing agent and sample boiling (Fig. 3B). Normal saline (e.g., 0.15 M NaCl) or cytosolic buffer also produced separate profilin peaks (not shown), but the peaks were <10% of the heights as those shown in Fig. 3, presumably due to loss of protein by precipitation or detection problems inherent with CZE under high salt conditions (23,24). Regardless of any condition employed, some of the profilin

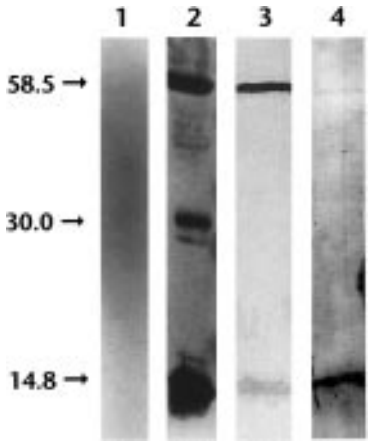

FIG. 2. Immunoblot analysis of profilin multimers. Human platelet profilin purified by different methods was separated by SDS-PAGE and transferred to nitrocellulose membranes. Immunoblots were developed by exposure to rabbit IgG anti-human platelet profilin and visualized by HRP-conjugated secondary antibody. *Lane 1* = actin (5 μg; antibody negative control); *lane 2* = total affinity column purified profilin (20 μg); *lane 3* = protein electroeluted from the initial 14.8 kD profilin band (5 μg); *lane 4* = similar to lane 3, but from a preparation in which 14.8 kD > 58.5 kD protein.

multimeric complex persisted. The data indicate a single profilin complex (presumably profilin$_4$) comprised of sulphydryl and/or ionic bonds exists in solution.

To examine if the higher order of profilin complexes with actin, transfer membranes containing profilins were incubated with human platelet G-actin at a concentration (3 μM) $\geq$ the apparent affinity for profilin (Fig. 4). Surprisingly, actin bound only to profilin$_4$, whereas profilin$_1$-actin complexes were not detected. When a blot containing profilin$_1$ $\geq$ profilin$_4$ was employed, no actin-profilin$_1$ binding was evident. In addition, extended exposure of profilin blots to actin (>2 hrs) resulted in a faint band associated with profilin$_1$ in only 10% of the blot overlays (not shown), suggesting either a low affinity of profilin$_1$ for actin, or that some profilin$_4$ may form from monomers during the membrane transfer step. Chicken muscle actin likewise bound only to profilin$_4$ (not shown), which was expected given the high degree of homology among most of the known actins. The data suggest that actin preferentially binds to profilin$_4$, and profilin$_4$-actin binding is not a phenomenon unique to the platelet system.

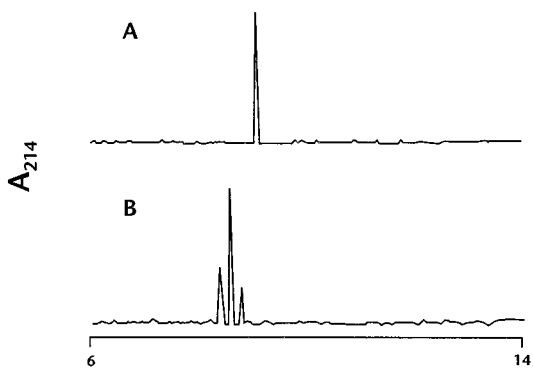

FIG. 3. Capillary zone electrophoresis of purified profilin. Electroelution purified profilin was injected by hydrostatic mode and run as described in Methods: (A) Non-reducing conditions, (B) addition of DTT (10 mg/ml) and sample boiling. The tracings are representative of at least three different sample runs.

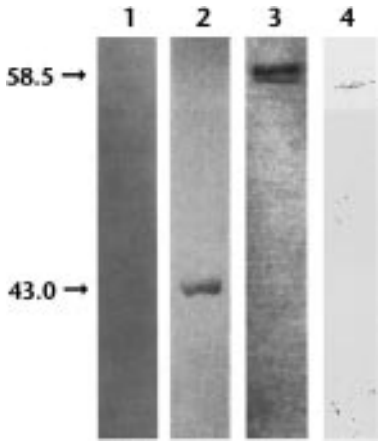

FIG. 4. Actin-binding to profilin immunoblot. Proteins (10 μg/lane) were run on SDS-PAGE, transferred to nitrocellulose membranes, exposed to G-actin and/or anti-actin antibodies, and visualized by HRP-conjugated secondary antibodies. *Lane 1* = transfer membrane containing profilin alone (negative control); *lane 2* = actin immunoblot (antibody positive control); *lane 3* = transfer membrane containing profilin incubated with G-actin then exposed to anti-actin antibodies; *lane 4* = similar to lane 3, but from a preparation in which 14.8 kD > 58.5 kD protein.

DISCUSSION

The results demonstrate that profilin$_1$ forms multimers and that profilin$_4$ is conceivably the high affinity actin-binding form. Furthermore, the relative difference in the amount of multimers resulting from gel slice vs. electroeluted profilin$_1$ (Fig. 1, lane 3 vs. 4) suggests that profilin$_4$ represents the lowest free-energy form. Re-electrophoresis of gel slices apparently does not permit sufficient time to form multimer orders higher than a dimer, whereas tetramers become more prevalent with the longer electroelution and purification process ($\geq$6 hrs). In support of the preferred formation of profilin$_4$, measurements of dynamic elastic and viscosity properties of profilin in solution showed at steady-state ($\geq$5 hrs) a viscoelastic material was formed (28). Thus, profilin resembles other non-filamentous materials by exhibiting both solid- and liquid-like properties and was suggested to form floccules (aggregates). The present work elucidates further on this suggestion by demonstrating distinct profilin$_4$ complexes are formed rather than floccules consisting of randomly associated profilin$_1$.

In the context that profilin-actin interactions occur in a 1:1 ratio (1,2,10), the present study suggests the ratio may represent formation of profilin$_4$-4actin. The idea of multiple actins and profilins forming complexes is consistent with the resolved three-dimensional structure of crystalline profilin-β-actin (25) which demonstrates two profilin contact sites within an actin ribbon structure. Furthermore, profilin:actin crystals (25–27) display actin ribbons that are stacked and held in place by profilin molecules. Our data extend those observations and suggest profilin$_4$ forms the intermolecular network to elicit stacked actin ribbons by [profilin$_4$-4actin]$_n$ complexes, although [profilin$_4$-or profilin$_1$-1actin]$_n$ complexes cannot be ruled out.

The present work may provide a molecular basis to address discrepancies on how different profilin preparations vary in their ability to not only bind actin (3,8,29), but to either inhibit or enhance barbed-end elongation (2–4,13,14). Perhaps some of the discrepancies on profilin function stem from the protein isolation methods employed. For instance, previous reports indicate that cellular levels of profilin (8) are insufficient to support a predominant G-actin sequestering role. However, the cellular profilin levels may be underestimated because it is presently unclear whether endogenous profilin$_4$ binds to standard affinity columns used for actin and profilin isolation. In this

regard, multimers found in the initial (i.e., total) profilin preparations may have derived from poly(l-proline)-sepharose 4B column-eluted monomers that ultimately form profilin$_4$. In addition, the association of profilin with actin is often observed by co-eluting the two proteins from affinity columns followed by reducing conditions with SDS-PAGE separation to yield different bands upon Coomassie Blue protein visualization. Thus any profilin$_4$-actin vs. profilin$_1$-actin complexes would not be discerned under those conditions. A further complexity is that the size of profilin$_4$ (58.5 kD) is fortuitously near the predicted size of a profilin$_1$-1actin complex. Therefore, profilin$_4$ identified by standard electrophoresis procedures could be associated with what is considered to be profilin$_1$-1actin.

The regulation of profilin tetramerization and kinetic analysis on actin binding requires further study. For instance, we sometimes observed differences in the relative amounts of profilin$_4$:profilin$_1$ between preparations. One possibility is that different forms of profilin are expressed with widely different isoelectric points (e.g., 30,31) that may complex together with strong ionic bonds at physiologic pH to withstand SDS-PAGE and harsh reducing conditions (32). Further work is also necessary to verify the functional role of profilin$_4$ in living cells and to determine the significance of monomeric profilin.

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