Detergent-resistant membranes contain signaling and integral membrane proteins that organize cholesterol-rich domains called lipid rafts. A subset of these detergent-resistant membranes (DRM-H) exhibits a higher buoyant density (~1.16 g/ml) because of association with membrane skeleton proteins, including actin, myosin II, myosin 1G, fodrin, and an actin- and membrane-binding protein called supervillin (Nebi, T., PestiIonamasp, K. N., Leszyk, J. D., Crowley, J. L., Oh, S. W., and Luna, E. J. (2002) J. Biol. Chem. 277, 43399–43409). To characterize interactions among DRM-H cytoskeletal proteins, we investigated the binding partners of the novel supervillin N terminus, specifically amino acids 1–830. We find that the supervillin N terminus binds directly to myosin II, as well as to F-actin. Three F-actin-binding sites were mapped to sequences within amino acids 280–342, 344–422, and ~700–830. Sequences with combinations of these sites promote F-actin cross-linking and/or bundling. Supervillin amino acids 1–174 specifically interact with the S2 domain in chicken gizzard myosin and nonmuscle myosin IIA (MYH-9) but exhibit little binding to skeletal muscle myosin II. Direct or indirect binding to filamin also was observed. Overexpression of supervillin amino acids 1–174 in COS7 cells disrupted the localization of myosin IIB without obviously affecting actin filaments. Taken together, these results suggest that supervillin may mediate actin and myosin II filament organization at cholesterol-rich membrane domains.

Compartmentalized signaling involving cholesterol-rich, liquid-ordered membrane domains occurs during cell activation triggered by receptor cross-linking, growth factors, or other extracellular stimuli (1–3). The redistribution of similar liquid-ordered domains, called lipid “rafts,” accompanies and is required for cell polarization and directed migration (4–8). Although we do not know the precise molecular mechanisms by which the redistributions of plasma membrane domains occur, an active role of the actin-based membrane skeleton has long been postulated (reviewed in Ref. 9).

Redistributions of activated or cross-linked receptors are accompanied by corresponding changes in the localizations of actin, nonmuscle myosin II, spectrin, and associated cytoskeletal proteins (9). Furthermore, disruption of actin filament integrity inhibits many lipid raft-mediated processes, including epidermal growth factor receptor capping in A431 cells (10), insulin receptor capping in lymphocytes (11), activation of fibroblasts (12), polarization of T lymphocytes (5), and down-regulation of FcεRI-mediated signaling in mast cells (13). A requirement for myosin II is shown by the greatly diminished receptor redistributions and/or developments of cell polarity that have been observed in cells that either lack myosin II (14, 15) or express a dominant-negative mutant of myosin II function (16–18). Erythrocyte spectrin (19) and the nonerythroid spectrin called fodrin 1 (20, 21) also have been implicated in lipid raft-mediated processes. Taken together, these observations suggest that actin filaments, perhaps as components of a spectrin-based membrane skeleton, are required for the anchorage of many receptors to lipid rafts, or as tracks for myosin-driven lateral movements of membrane proteins during signaling, or as part of both processes.

Lipid raft components may be isolated by flotation into low buoyant density sucrose fractions after treatment of cells or membranes with cold Triton X-100 (3). During this procedure, the ~50-nm liquid-ordered domains present in unactivated cells (22, 23) coalesce into detergent-resistant membranes (DRMs) that represent a subset of the endogenous raft lipids and proteins (2, 3). Both lipid rafts and DRMs contain resident integral membrane proteins, such as caveolin, stomatin, and flotillin, and signaling proteins, including heterotrimeric G proteins and members of the Src family of protein-tyrosine kinases (24).

Raft-associated integral membrane and signaling proteins in bovine neutrophil plasma membranes sediment in sucrose gradients as both “light” (DRM-L) and “heavy” (DRM-H) fractions with buoyant densities of ~1.09–1.13 and ~1.15–1.18 g/ml, respectively (25). The neutrophil DRM-H fraction also contains a subset of cytoskeletal proteins, including actin, myosin II, fodrin, α-actinins 1 and 4, vimentin, myosin 1G, and the actin-binding protein supervillin. Most of the integral, signaling, and cytoskeletal proteins in the DRM-H fraction continue to co-
sediment with each other after solubilization of raft lipids with octylglucoside, indicating that these proteins are associated through interactions exclusive of those with the bilayer. Supervillin, myosin II, and myosin 1G remain bound to the bilayer after a high pH carbonate extraction, suggesting that these proteins are more proximal to the membrane than are the other DRM-H cytoskeletal proteins. Thus, the DRM-H fraction consists of an actin- and fodrin-based membrane skeleton that is associated with a subset of lipid raft signalling domains, possibly through interactions with supervillin and myosin.

Similar raft-associated membrane skeletons may be present in many cell types. Supervillin is a constituent of total lipid rafts from Jurkat T cells (26) and is purified from HEK293 cells in many cell types. Supervillin is a constituent of total lipid bilayers through interactions with supervillin and myosin. Associated with a subset of lipid raft signalling domains, possibly with other proteins that are more proximal to the membrane than are the other DRM-H cytoskeletal proteins. Thus, the DRM-H fraction consists of an actin- and fodrin-based membrane skeleton that is associated with a subset of lipid raft signalling domains, possibly through interactions with supervillin and myosin.

Supervillin, so-named because of C-terminal similarities to the microvillar protein villin, binds directly and specifically to F-actin (29, 30) and localizes to sites of cell-cell and cell-substrate adhesion in epithelial cells (30, 31). Archvillin localizes at costameres, specialized adhesion sites in muscle (28). The shared N terminus of supervillin and archvillin is novel, lacking any similarity to the S1 domain in villin and gelsolin that is involved in actin filament severing activity (32–34). Instead, the supervillin N terminus contains functional nuclear localization sequences and F-actin binding and bundling activities (31). Overexpression of supervillin or its N terminus disrupts stress fibers and vinculin-containing focal adhesions (31), suggesting a role in the regulation of cell-substratum interactions. Dysfunction caused by overexpression of supervillin is supported by the increased levels of this protein found in many carcinoma cell lines (35) and by the demonstration that increased levels of supervillin can activate signaling through the androgen receptor (36).

To understand better the role of supervillin at the membrane, we are mapping functional domains. Here we report the presence and localizations of a binding site for myosin II and three binding sites for filamentous actin within the supervillin N terminus. These F-actin-binding sites support filament bundling and cross-linking in vitro and thus can account for the observed aberrations in F-actin distributions documented in vivo (31). The myosin II-binding site, which is also present in archvillin, selectively recognizes nonmuscle and smooth muscle myosin II isoforms, as opposed to skeletal muscle myosin II. This selectivity is consistent with the co-localization of archvillin with nonmuscle myosin II at the sarcolemma in differentiating and mature skeletal muscle (28). Overexpression of the myosin II-binding sequence disrupts the co-localization of nonmuscle myosin II with actin filament bundles in COS7 cells, suggesting a role for supervillin, and by extension, archvillin, in the organization of actin and myosin II at liquid-ordered membrane domains.

### EXPERIMENTAL PROCEDURES

#### Materials

Glutathione-Sepharose™, PreScission™ Protease, and DEAE-Sepharacy™ were purchased from Amersham Biosciences. Chicken gizzards were from Pel-Freeze Biologicals (Rogers, AR). Chemical reagents were from Sigma, Calbiochem-Novabiochem, Fisher, or VWR International Inc. (Grovel, IL).

#### cDNAs

**EGFP-Supervillin**—Bovine supervillin cDNA (NCBI Nucleotide Database accession number AF025996) was used as a template to generate by PCR chimeric cDNAs from forward and reverse primers with 5’ restriction enzyme sites for cloning into pGEM-T (Promega Corp., Madison, WI), pGEM-T Easy (Promega), or pCR2.1-TOPO® (Invitrogen) TA vectors. Primer and/or vector cloning sites were used to transfer supervillin sequences into the pGEX-6P-1 vector (Amersham Biosciences) for expression as fusion proteins with glutathione S-transferase (GST) and into pEGFP vectors (Clontech, Palo Alto, CA) for mammalian cell expression (Table I). The construction of EGFP-SV C-terminal deletions (HMM 1, Met1-Ser1110), and myosin subfragment-2 (S2, Leu581-Glu1106) were also cloned into the pEGFP-N1 vector. Proteins were expressed in Sf9 cells. Essential and regulatory light chains were co-expressed and co-purified in association with HMM, HMM, HMM, and S1.

#### Proteins

**GST Fusions**—GST fusion proteins were expressed in BL21 cells and purified with glutathione-Sepharose™ (37). After cleavage of GST with PreScission™ Protease, the proteins were further purified by chromatography on DEAE-Sepharacy™ and elution with 0–0.2 M NaCl. The supervillin fragments were dialyzed against dialysis buffer (100 mM KCl, 2 mM MgCl2, 1 mM DTT in either 40 mM Pipes, pH 7.0, or 40 mM MOPS, pH 7.5). Dialyzed proteins were frozen quickly in liquid nitrogen and stored at −80 °C until use.

**Muscle Proteins**—G-actin was prepared from an acetone powder of rabbit skeletal muscle (38). G-actin was used directly in viscosity measurements or was column-purified (39) for use in co-sedimentation experiments and 125I-labelled G-actin blot overlays (40, 41). G-actin was stored at −7.0, or 40 mM MOPS, pH 7.5). Dialyzed proteins were frozen quickly in liquid nitrogen and stored at −80 °C until use.

### Table I

<table>
<thead>
<tr>
<th>Construct</th>
<th>pEGFP vector</th>
<th>Forward primer</th>
<th>Reverse primer</th>
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<tr>
<td>SV-(1-174)</td>
<td>N3</td>
<td>5′-GGATTCAUGAAGGGAAGGATGCCC-3′</td>
<td>5′-GGATCCCAACGCTGTCGAGGGAAG-3′</td>
</tr>
<tr>
<td>SV-(1-342)</td>
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<td>5′-GGATTCAUGAAGGGAAGGATGCCC-3′</td>
<td>5′-GGATCCCAACGCTGTCGAGGGAAG-3′</td>
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<tr>
<td>SV-(171-571)</td>
<td>C1</td>
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<td>5′-GGATCCCAACGCTGTCGAGGGAAG-3′</td>
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<tr>
<td>SV-(343-830)</td>
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<td>5′-GGATCCCAACGCTGTCGAGGGAAG-3′</td>
</tr>
<tr>
<td>SV-(570-830)</td>
<td>N2</td>
<td>5′-GGATTCAUGAAGGGAAGGATGCCC-3′</td>
<td>5′-GGATCCCAACGCTGTCGAGGGAAG-3′</td>
</tr>
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**DNA Sequencing**—All PCR products in TA vectors were verified by sequencing at the Iowa State University DNA Sequencing and Synthesis Facility (Ames, IA) or at the University of Massachusetts Nucleic Acid Facility (Worcester, MA). Expression constructs were checked by sequencing through the cloning sites.
Actin and Myosin Binding to Supervillin

Cell Culture and Transfection

COST-2 cells were grown in Dulbecco’s modified Eagle’s high glucose media supplemented with 10% fetal calf serum and transfected by electroporation (48) or with calcium phosphate-precipitated DNA (49). For analysis of EGFP-tagged supervillin sequences by F-actin blot overlay and anti-EGFP staining, transfected cells were washed with PBS (pH 7.4) and harvested from 10-cm plates using 0.5 mlplate of M-PER® Mammalian Protein Extraction Reagent, 3 mM pepstatin, 2 mM leupeptin, 0.2 μg/ml phenylmethylsulfonyl fluoride (PMSF), as described by the manufacturer (Pierce).

SDS-PAGE and Immunoblotting

Proteins were denatured by heating for 5 min at 95 °C in 2× SDS sample buffer and separated on SDS-polyacrylamide gels (50). Protein concentrations were determined by BCA Protein Assays™ (Pierce). Gels were stained for protein with Coomassie Blue or electrotransferred to nitrocellulose and blocked for 1 h at room temperature or overnight at 4 °C. Primary antibodies used in this study were diluted as follows: affinity-purified anti-supervillin (anti-H9262), 1:25; proteins resolved on 8% SDS-PAGE gels were electrotransferred to nitrocellulose and blocked for 1 h at room temperature or overnight at 4 °C. Antibodies were visualized using anti-mouse IgG, mouse IgM, or rabbit IgG antibodies conjugated to horseradish peroxidase, an ECL substrate kit (PerkinElmer Life Sciences). 35S-Labeled SV-(1–174) on glutathione-Sepharose was identified from in vitro transcription and translation of SV-(1–174) in the presence of [35S]methionine. Filters were washed five times for 1 h with 0.1% SDS and for another hour at room temperature. A PhosphorImager screen.

HS-174 fragment ions identified from MALDI Post-source Decay Mass Spectrometry (51) were subjected to mass spectrometric analysis and fragmentation at 270 × 5 × 4 °C and washed three times with 0.5 ml of PBS, 0.1% Triton X-100. In some experiments, beads were incubated at 4 °C for 20 min with higher stringency solutions containing 0.35 M NaCl, 0.5% NaCl, 1% Nonidet P-40, or 1% Nonidet P-40 + 0.5% SDS. Beads were then washed two more times with 0.1% Triton X-100, PBS before elution with glutathione. To investigate the interaction of double-headed myosin monomers with GST-tagged supervillin sequences, purified turkey gizzard myosin II or rabbit muscle HMM (0.5 mg in 0.5 ml) were incubated for 1 h at 4 °C with glutathione-Sepharose™ (50 μl) containing pre-bound GST or GST-supervillin proteins (100 μg) in binding buffer (10 mM MOPS, pH 7.5, 1 mM ATP, 5 mM MgCl2, 50 mM NaCl, 0.05% β-mercaptoethanol). Beads were transferred to a column, and the columns were washed with 0.5 ml of binding buffer and then with 100-μl portions of binding buffer containing increasing concentrations (0.1, 0.15, 0.2, and 0.3 mM) of NaCl. GST proteins and bound myosin were eluted from the washed columns with 250 μl of 5 mM glutathione in binding buffer. Fractions were collected and analyzed by Coomassie Blue staining of 6–20% gradient SDS-polyacrylamide gels.

For co-sedimentation experiments with purified myosin filaments or sedimentable myosin fragments, supervillin fragments (18 μg) were incubated for 10 min on ice with myosin II (100 μg of 1 mg/ml), rod (100 μg of 0.25 mg/ml), or LMM (100 μg of 0.4 mg/ml) in 50 mM KCl, 10 mM ATP, 10 mM MgCl2, 1 mM MgCl2, 1 mM ATP, 5 mM EGTA, 10 mM Tris-HCl, pH 8.0. The final concentrations of actin, GST fusion proteins, and GST were 2.3, ~2, and 3.5 μM, respectively. Actin filaments were centrifuged as above through 50 μl of 10% sucrose in actin polymerizing buffer. Supernatants were collected as the top 100 μl in each tube, the rest of the liquid was carefully removed, and pellets were resuspended to 150 μl with Buffer A. Equal volumes (30 μl) of supernatant and pellet fractions were loaded onto SDS-polyacrylamide gels and immunoblotted with anti-GST antibody or stained with Coomassie Blue.

Myosin Binding Assays

For initial binding experiments with GST fusion proteins, chicken gizzard filaments were disrupted and extracted with a low salt extract containing 2 mM Tris, 1 mM EDTA, 0.5 mM PMSF, pH 9.0 (57). Extracts were centrifuged at 12,000 × g for 10 min at 4 °C, neutralized with acetic acid, and stored in aliquots at −80 °C until use in sedimentation assays with GST and GST-SV-(1–174) on glutathione-Sepharose™ beads. Beads were pre-cleaned by centrifuging at 8000 × g for 15 min at 4 °C and then rotated for 6 h at 4 °C with glutathione-Sepharose™ beads (500 μl), 0.05% Triton X-100, 0.2 mg/ml PMSF and centrifuged at 500 × g for 5 min at 4 °C. The supernatant was divided, and aliquots were incubated separately with end-over-end rotation overnight at 4 °C with glutathione-Sepharose™ beads containing bound GST or GST-SV-(1–174). Beads were collected by centrifugation at 270 × 5 × 4 °C and washed three times with 0.5 ml of PBS, 0.1% Triton X-100. In some experiments, beads were incubated at 4 °C for 20 min with higher stringency solutions containing 0.35 M NaCl, 0.5% NaCl, 1% Nonidet P-40, or 1% Nonidet P-40 + 0.5% SDS. Beads were then washed two more times with 0.1% Triton X-100, PBS before elution with glutathione.
supervillin fragments were dialyzed against 40 mM PIPES, pH 6.9, 100 mM KCl, 2 mM MgCl₂, 1 mM DTT. Dialyzed supervillin fragments and G-actin in Buffer A were clarified by ultracentrifugation, as described above. The supervillin fragments (4 μg) were mixed on ice in 100-μl aliquots with 15 μg of G-actin into a final assay buffer containing 100 mM KCl, 40 mM PIPES, pH 6.9, 2 mM MgCl₂, 1 mM ATP, 1 mM DTT, 0.004% NaN₃, 0.1 mM EGTA, and 10 μM CaCl₂. Mixed samples were incubated at 28 °C for 60 s, washed for 15 s with 100 mM KCl, 2 mM MgCl₂, 10 mM PIPES, pH 7.0, 0.3 mM NaN₃, and stained for 15 s with freshly filtered 1% uranyl acetate in PBS. Grids were dried slowly and examined on a model EM-301 transmission electron microscope (Philips Electron Optics Inc, Rahway, NJ) at an accelerating voltage of 60 kV.

Actin and Myosin Binding to Supervillin

Supervillin fragments were analyzed using a reciprocating motor-driven constant-speed equipment for differential centrifugation. The supernatants were collected and the precipitates were dissolved in SDS sample buffer. The supernatants and precipitates were analyzed by 10% SDS-PAGE and Coomassie Blue staining. The precipitates were analyzed by 20% SDS-PAGE and Coomassie Blue staining. The results were analyzed using the ImageJ software (NIH).

Electron Microscopy

To characterize sequences within the supervillin N terminus responsible for binding to F-actin (29, 31, 61), we generated constructs encoding subsets of the N-terminal 830 supervillin amino acids (SV-(1–830); Fig. 1). To identify likely boundaries between functional domains, we aligned predicted protein sequences from cDNA and genomic data bases, and we identified regions of low sequence conservation among vertebrates. These candidate regions were further analyzed for the presence of predicted secondary structures using the algorithms available on the Jpred server (www.compbio.dundee.ac.uk/~www-jpred/submit.html) (62). Bovine supervillin residues 171–174, 342–344, and 570 were chosen as probable inter-domain boundaries because these sequences correspond to predicted protein loops with relatively low interspecies conservation. Together,
they permitted the supervillin N terminus to be subdivided into four regions of approximately equal size (Fig. 1). Each of these four regions and each combination of adjacent regions were generated by PCR, expressed in COS7 cells as fusions with EGFP (see below), expressed in Escherichia coli as GST fusions (Fig. 1), cleaved, and purified (Fig. 1). The four smallest fragments (SV-(1–174), SV-(171–342), SV-(343–571), and SV-(570–830)) could be purified to >90% (Fig. 1C, lanes 1–4). Larger fragments were isolated with variable amounts of truncated protein products (Fig. 1C, lanes 5–7). Bacterially expressed proteins containing more than 60 kDa of supervillin sequence were mostly degraded (not shown).

Identification of F-actin-binding Sites—Three of the four

![Image](http://www.jbc.org/)

**Fig. 2.** F-actin binds independently and directly to three regions of supervillin (SV-(171–342), SV-(343–571), and SV(570–830)). A, separation of individual purified fragments of supervillin (0.18 mg/ml), as shown in Fig. 1C, into supernatants (S) and pellets (P) after sedimentation in the presence (+) or absence (−) of pre-polymerized F-actin (1.0 mg/ml), pH 7.5. All proteins were detected by SDS-PAGE and staining with Coomassie Blue, except for SV-(1–174), which was detected with anti-H340 antibodies due to co-migration with actin. All fragments, except for SV-(1–342), sediment with actin filaments to some degree; no supervillin fragments sediment in the absence of F-actin. B, blot overlays with 125I-labeled F-actin of lysates from COS7 cells transfected with EGFP fusions with supervillin sequences. After protein resolution by SDS-PAGE and electrolytic transfer to nitrocellulose, identical loaded blots were probed with either antibody against EGFP (left) or 125I-labeled F-actin (right). C, anti-GST immunoblots showing that only nearly full-length GST-SV-(171–342) and GST-SV-(343–571), co-sediment with F-actin at pH 8.0 (top panel). No sedimentation of any GST-tagged protein was observed in the absence of actin (bottom panel). FL, locations of full-length proteins; GST, location of GST with no C-terminal supervillin sequences.

**Fig. 3.** Viscosities of solutions containing F-actin and supervillin sequences. Viscosity, in centipoise (cp), as a function of the concentration of the supervillin fragments SV-(1–342), SV-(171–571), and SV-(343–830). G-actin (0.5 mg/ml) was added to purified supervillin fragments and polymerized for 1 h at 28 °C before a low shear assay for viscosity. Inclusion of SV-(171–571) (△) and SV-(343–830) (■), but not SV-(1–342) (○), resulted in increased viscosities of mixtures containing polymerized actin.

**Fig. 4.** Effects of supervillin sequences on actin filament organization. Electron micrographs of negatively stained actin filaments (150 μg/ml) polymerized in the absence (A) and in the presence of purified supervillin fragments (40 μg/ml), SV-(1–342) (B), SV-(171–571) (C), or SV-(343–830) (D). The mixture also contained 100 mM KCl, 40 mM PIPES, pH 6.9, 2 mM MgCl2, 1 mM ATP, 1 mM DTT, 0.004% NaN3, 10 μM CaCl2, 0.1 mM EGTA. Large bundles of actin filaments are observed in the presence of SV-(343–830). Bar, 200 nm.
subdomains of the supervillin N terminus (SV-(171–342), SV-(343–571), and SV-(570–830)) bound F-actin. Each of these purified proteins co-sedimented with actin filaments in vitro (Fig. 2A, left panels, lane 2). Binding was enhanced by the presence of two actin-binding sites. Proteins containing combinations of two smaller actin-binding domains (SV-(171–571), SV-(343–830)) were almost completely pelleted with F-actin in this assay (Fig. 2A, right panels, lane 2). By contrast, no detectable SV-(1–174) was observed in pellets with F-actin. SV-(1–342) was only partially sedimented (Fig. 2A, lane 2), consistent with the presence of a single F-actin-binding site. Thus, the supervillin N terminus contains at least three sites capable of binding to actin filaments.

F-actin blot overlays of lysates from COS7 cells expressing fusion proteins containing EGFP and supervillin sequences supported the assignment of an F-actin-binding site in each of these three supervillin sequences (Fig. 2B). EGFP fusion proteins on nitrocellulose blots after SDS-PAGE and electrotransfer were identified with antibodies against EGFP (Fig. 2B, left panel) and probed with 125I-labeled F-actin (Fig. 2B, right panel). Although no radioactivity was observed in association with EGFP (Fig. 2B, lanes 1) or with EGFP-SV-(1–174) (Fig. 2B, lanes 2), EGFP-SV-(171–342), EGFP-SV-(343–571), and EGFP-SV-(570–830) each bound directly to 125I-labeled F-actin (Fig. 2B, lanes 3–5). By contrast, no binding was observed to SV-(423–571) tagged with EGFP at the C terminus (data not shown), suggesting that amino acids 342–422 are required for F-actin binding to this supervillin fragment in the blot overlay assay.

By taking advantage of the proteolysis or incomplete translation of bacterially expressed chimeric proteins containing N-terminal GST and C-terminal supervillin sequences, we used partially purified GST proteins to map further the approximate locations of the F-actin-binding sites within each supervillin fragment (Fig. 2C). Supernatants and pellets after sedimentation of each of these proteins with actin filaments were analyzed by immunoblotting with antibodies against GST. The sizes of the GST-tagged polypeptides that did, and did not, bind to F-actin were estimated to approximate the location of the actin-binding site(s) within each of the GST-tagged supervillin fragments. Polypeptides with both N-terminal GST and a C-terminal actin-binding site co-pelleted with F-actin (Fig. 2C, lanes 3); shorter N-terminally tagged GST fusion proteins that had lost the F-actin-binding site from their C termini were enriched in the F-actin supernatants (Fig. 2C, lanes 5).

As expected, full-length GST-SV-(171–342), GST-SV-(343–571), and GST-SV-(570–830) co-sedimented with F-actin (Fig. 2C, top panel, lanes 6, 8, and 10), but GST (Fig. 2C, top panel, lane 2) and GST-SV-(1–174) (Fig. 2C, top panel, lane 4) did not. F-actin pellets were enriched in full-length GST-SV-(171–342) relative to a truncated protein that lacked −7 kDa of C-terminal sequence (Fig. 2C, top panel, lane 6), suggesting that the missing sequences are important for actin binding activity. Similarly, full-length GST-SV-(570–830) (Fig. 2C, top panel, lane 10), but not the next smaller related GST-tagged polypeptides (Fig. 2C, top panel, lane 9), sedimented with actin filaments, indicating that the C-terminal −7 kDa of sequence in this domain is essential for binding to F-actin. By contrast, essentially all truncated GST fusion proteins containing SV-(343–571) sequences were pelleted (Fig. 2C, top panel, lane 8), consistent with the presence of an F-actin-binding site near the N terminus of this fragment. Thus, the supervillin N terminus apparently contains F-actin-binding sites in the vicinity of amino acids −280–342, −344–422, and −700–830. Supervillin-induced Changes in Actin Filament Organization—To determine whether supervillin N-terminal F-actin-
binding sequences can directly induce changes in actin filament organization, we measured the low shear viscosity of solutions containing actin filaments and supervillin proteins predicted to contain one (SV-(1–342)) or two (SV-(171–571) and SV-(343–830)) binding sites for F-actin (Fig. 3). The low shear viscosity of a solution containing F-actin and associated proteins is very sensitive to the geometry and extent of the resulting structures, permitting sensitive detection of changes in actin organization (63). Solutions containing F-actin polymerized in the presence of SV-(1–342) exhibited approximately the same viscosities as a solution containing F-actin alone (Fig. 3, open circles), indicating that final filament lengths and filament organization were essentially unaffected by these sequences. By contrast, inclusion of SV-(171–571) induced large increases in solution viscosity (Fig. 3, open triangles), a profile reminiscent of observations made for F-actin cross-linking proteins (63). SV-(343–830) induced an ~3-fold increase in viscosity at ~100 μg/ml but resulted in viscosities that were even less than that of actin alone at higher concentrations of SV-(343–830) (Fig. 3, closed squares). This pattern of viscosity changes was similar to that reported for the F-actin bundling protein, α-actinin (64), raising the possibility that SV-(343–830) also might induce actin filament bundling.

Solutions of F-actin polymerized in the presence of supervillin sequences also were examined for gross changes in organization by negative-stain electron microscopy (Fig. 4). Filament organization was not obviously affected by the presence of SV-(1–342) (Fig. 4, B versus A) or SV-(171–571) (Fig. 4C). However, actin filaments were noticeably bundled in solutions containing SV-(343–830) (Fig. 4D). Thus, the actin-binding sites present in SV-(343–830) are sufficient for the formation of actin filament bundles.

To determine the effects of the supervillin N-terminal actin-binding sites on actin filament organization in vivo, we overexpressed each of the sequences under study here as an EGFP fusion protein in COS7 cells (Fig. 5). Consistent with expectations based on viscosity assays and electron microscopic observations, EGFP-tagged SV-(1–342) was more closely associated with microfilaments (Fig. 5d) than was EGFP alone (Fig. 5a) but did not obviously affect the organization of phallodin-stained basal microfilament bundles (Fig. 5, e versus b, arrows). In agreement with the prediction that they should each contain two F-actin-binding sites, EGFP-SV-(171–571) and EGFP-SV-(343–830) co-localized strongly with actin filaments. EGFP-SV-(171–571) associated with F-actin in short cell surface extensions (Fig. 5, c versus b, arrows), and increased the prevalence of these structures (Fig. 5h, arrowhead), at the apparent expense of basal microfilaments. EGFP-SV-(343–830) (Fig. 5, j–l) localized in similar surface extensions and increased the amount and extent of microfilament bundles around the cell peripheries (Fig. 5k, arrowheads).

Surprises arose from the localizations of the shortest subdomains of the supervillin N terminus. Based on the absence of binding to F-actin in vitro (Fig. 2), EGFP-SV-(1–174) was expected to lack an association for microfilaments. However, this supervillin subdomain was consistently found along microfilaments and/or at microfilament-associated punctae (Fig. 5, m–o). EGFP-SV-(171–342), EGFP-SV-(343–571), and EGFP-SV-(570–830) were predicted to associate with actin filaments but to have marginal, if any, effects on cytoskeletal organization. This prediction was largely true for EGFP-SV-(171–342) (Fig. 5, p–q) and EGFP-SV-(570–830) (Fig. 5, v–x), and EGFP-SV-(343–571) was associated with peripheral F-actin structures (Fig. 5, s–u, arrowheads). However, moderate overexpression of EGFP-SV-(343–571) (Fig. 5, s–u) decreased the percentage of transfected cells with basal microfilament bundling to ~35% from the 92–95% observed for cells expressing EGFP alone or with most of the other supervillin fragments (Fig. 5, arrow). EGFP-SV-(171–342) also may have induced a modest decrease in the numbers of basal microfilaments, but at least 82% of the COS7 cells transfected with EGFP-SV-(171–342) contained at least some of these structures. Taken together, these results suggested the presence of cytoskeleton-associated binding sites in the supervillin N terminus in...
Actin and Myosin Binding to Supervillin

The most striking of these results was the strong correlation between basal microfilament bundles and structures containing SV-(1–174), a sequence that lacks detectable F-actin binding activity.

Filamin and Myosin II Binding to SV-(1–174)—To identify potential binding partners for SV-(1–174) that might explain the localization of this sequence with microfilaments in vivo (Fig. 5, m–o), we fractionated a low salt extract of chicken gizzard smooth muscle (57) by affinity chromatography on columns containing GST-SV-(1–174) or GST alone (Fig. 6A). Polypeptides with molecular masses of ~250 and ~210 kDa were prominent in fractions specifically eluted from GST-SV-(1–174) columns (Fig. 6A, lane 4) but did not bind to columns containing GST alone (Fig. 6A, lane 2).

The ~250- and ~210-kDa polypeptides that bound specifically to SV-(1–174) sequences were identified as filamin and smooth muscle myosin II heavy chain, respectively (Fig. 6, B and C). Database searches with peptide masses obtained by MALDI-TOF mass spectrometry from tryptic digests of these two polypeptides matched well with masses predicted for GalIus galls filament (NCBI Protein Database accession number BAB63943) and smooth muscle myosin II heavy chain (MYHB, NCBI Nucleotide Database accession number X06546).
Myosin II heavy chain bind well to SV-(1–174) on blot overlays. and HMM/H9004), myosin subfragment-2 (HMM), rod S1/H9004), myosin subfragment-1 (HMM), and chymotryptic light meromyosins (LMM), and truncated heavy meromyosins (HMM). Boundary amino acid residues for each truncation are L1108 in chicken smooth muscle myosin heavy chain (MHC, NCBI Protein Database accession number P10587), myosin fragments used in this study: full-length smooth muscle myosin heavy chain (MHC, NCBI Protein Database accession number P10587), heavy meromyosin (HMM), truncated heavy meromyosins (HMMΔ1, HMMΔ2), myosin subfragment-1 (S1), myosin subfragment-2 (S2), rod generated by digestion with papain (Rod), and chymotryptic light meromyosin (LMM). Boundary amino acid residues for each truncation are indicated. All proteins, except rod and LMM, were purified from insect cells overexpressing chicken gizzard smooth muscle myosin heavy chain sequences.

of these matches are provided in the Supplemental Material Table II. Molecular weight search scores were highly significant (Fig. 6B), and tandem mass spectrometry (MS/MS) via MALDI (PSD) of selected peptides confirmed the presence of filamin and smooth muscle myosin II sequences (Fig. 6B, Supplemental Material Table II).

The presence of filamin and myosin II in fractions eluted specifically from GST-SV-(1–174) was further confirmed by immunoblot analyses of the −250- and −210-kDa polypeptides (Fig. 6C). As described originally (57), filamin and myosin II were present in the low salt gizzard extract (Fig. 6C, lane 1). Filamin and myosin II also bind to the GST-SV-(1–174) column (Fig. 6C, lane 2) but not to columns containing GST alone (Fig. 6C, lane 3). Although detectable in the gizzard extract, no actin could be detected in the fractions eluting with glutathione from either GST-SV-(1–174) or GST columns (Fig. 6C). Thus, although this assay could not differentiate between direct and indirect binding of filamin and myosin II to GST-SV-(1–174), any indirect associations were not caused by mutual binding to F-actin.

**Direct Binding to Smooth Muscle Myosin II**—A single polypeptide of −210-kDa in gizzard extracts and in glutathione-eluted fractions from GST-SV-(1–174) was recognized by 35S-labeled SV-(1–174) in a blot overlay assay (Fig. 7A). Given the presence of significant amounts of the similarly sized myosin heavy chain in these fractions (Fig. 6), we used purified gizzard muscle myosin in a “pulldown” assay with glutathione-Sepharose™ beads and either GST or GST-SV-(1–174) (Fig. 7B). Smooth muscle myosin co-sedimented with beads containing bound GST-SV-(1–174) (Fig. 7B, lanes 2, 4, 6, and 8) but not to beads with GST only (Fig. 7B, lanes 1, 3, 5, and 7). The interaction appeared to be of relatively high avidity because myosin binding was retained after washing with increased levels of salt (Fig. 7B, lanes 2 and 4) or detergent (Fig. 7B, lanes 6 and 8). However, increased salt did lower the amount of bound myosin heavy chain (Fig. 7B, lanes 2 and 4), raising a
supervillin fragments co-sedimented with filaments composed of smooth muscle myosin (Fig. 7D, lanes 2 and 8), but neither SV-(1–174) (not shown) nor SV-(1–342) (Fig. 7D, lanes 10) bound to filaments composed of skeletal muscle myosin II. These results indicated that SV-(1–174) could bind to smooth muscle myosin II filaments, as well as to monomers.

To test whether double-headed monomeric skeletal muscle myosin II might be capable of binding to supervillin sequences, we performed GST pulldown assays with skeletal muscle HMM (Fig. 7E). Under conditions identical to those that supported binding of smooth muscle myosin monomers (Fig. 7C), we found that ≤0.001 mol of skeletal muscle HMM bound per mol of either GST-SV-(1–174) or GST-SV-(1–342) (Fig. 7E, lanes 5–7). The lack of significant binding of either filamentous (Fig. 7D) or monomeric (Fig. 7E) skeletal muscle myosin II to supervillin N-terminal sequences suggests that the binding observed for filamentous (Fig. 7B) and monomeric (Fig. 7C) smooth muscle myosin II may represent an isoform-specific interaction.

To determine which region of smooth muscle myosin II interacts with supervillin sequences, we examined the association of SV-(1–174) with myosin fragments (Figs. 8 and 9). Most of these fragments (HMM, HMMΔ1, HMMΔ2, S1, and S2) were purified after expression in insect cells, as described previously (47). Two fragments (rod, LMM) were purified after proteolytic digestion of turkey gizzard myosin (45, 46). Fig. 8A shows a diagram of these myosin fragments and the residues at which their sequences were truncated. Blot overlays with 35S-labeled SV-(1–174) showed that HMM, HMMΔ1, and HMMΔ2 bound directly to SV-(1–174) (Fig. 8B, bottom panel, lanes 1, 2, 4, and 5). Binding to HMMΔ1 (lane 4) and, especially, HMMΔ2 (lane 5) was perhaps reduced slightly, as compared with HMM (lanes 1 and 2), but some variability in binding was observed relative to the amounts of loaded proteins (Fig. 8B, bottom panel versus top panel, lanes 1). Also, very little binding was observed to myosin S1 that had been denatured and processed identically with HMM (Fig. 8B, bottom panel versus top panel, lanes 1). Binding of SV-(1–174) with myosin fragments (Figs. 8 and 9). Most of these fragments (HMM, HMMΔ1, HMMΔ2, S1, and S2) were purified after expression in insect cells, as described previously (47). Two fragments (rod, LMM) were purified after proteolytic digestion of turkey gizzard myosin (45, 46). Fig. 8A shows a diagram of these myosin fragments and the residues at which their sequences were truncated. Blot overlays with 35S-labeled SV-(1–174) showed that HMM, HMMΔ1, and HMMΔ2 bound directly to SV-(1–174) (Fig. 8B, bottom panel, lanes 1, 2, 4, and 5). Binding to HMMΔ1 (lane 4) and, especially, HMMΔ2 (lane 5) was perhaps reduced slightly, as compared with HMM (lanes 1 and 2), but some variability in binding was observed relative to the amounts of loaded proteins (Fig. 8B, bottom panel versus top panel, lanes 1). Also, very little binding was observed to myosin S1 that had been denatured and processed identically with HMM (Fig. 8B, bottom panel versus top panel, lanes 1). Binding of SV-(1–174) with myosin fragments (Fig. 8B, bottom panel, lanes 1, 3, and 5) shows a slight difference in binding between the two panels. These results indicated that SV-(1–174) can bind to myosin fragments that lack the N-terminal sequences, but not by other EGFP-tagged supervillin constructs. Bar, 10 μm.

To determine whether double-headed smooth muscle myosin II monomers could bind to supervillin sequences in GST pull-down assays, turkey gizzard myosin in a buffer containing 1 mM MgATP was incubated with glutathione-Sepharose™ beads containing GST or GST chimeric proteins with supervillin sequences (Fig. 7C). Monomeric smooth muscle myosin II bound to columns containing GST-SV-(1–174) or GST-SV-(1–342), even after extensive washing with high salt solutions (Fig. 7C, lanes 7). By contrast, no significant association of myosin to GST-SV-(171–342), GST-SV-(343–571), or GST-SV-(570–830) was observed. Thus, supervillin residues 1–174 are sufficient for binding to monomeric smooth muscle myosin II.

Sedimentation assays with smooth and skeletal muscle myosins were used to determine whether SV-(1–174) and SV-(1–342) bind to filamentous myosin II (Fig. 7D). Both of these

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To determine whether the myosin S2 domain is sufficient for binding to SV-(1–174), we tested the binding of purified recombinant smooth muscle myosin S1 and S2 domains to GST fusion proteins in a glutathione-Sepharose pulldown assay (Fig. 9). Myosin S1 interacted only slightly with GST-SV-(1–174) (Fig. 9A, top panel, lanes 1–7) but not by other EGFP-tagged supervillin constructs. Bar, 10 μm.

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Actin and Myosin Binding to Supervillin

Myosin S2 was independently identified as a key interaction site for the supervillin N terminus in an untargeted yeast two-hybrid screen of a HeLa cDNA library that was probed with a bait vector encoding supervillin amino acids (11–174) and human nonmuscle myosin IIA (MYH-9, NCBI Protein Database accession number P35579) were obtained. These colonies supported growth on inductive, selective media and induced expression of β-galactosidase activity (Fig. 9B). Seven of these clones encoded amino acids 874–1095 in human MYH-9 (Fig. 9, B and C, 1), two encoded residues 874–1100 (Fig. 9, B and C, 2), and one encoded amino acids 846–1100 (Fig. 9, B and C, 3). The shortest sequence identified in this assay corresponds to residues 887–1108 in chicken gizzard smooth muscle myosin MYHB (Fig. 8A), based on a sequence alignment of these two proteins (see below). Thus, the untargeted yeast two-hybrid screen also identified sequences within myosin S2 as important interaction sites for the supervillin N terminus.

Co-localization of Nonmuscle Myosin II with Supervillin Sequences in Vivo—Consistent with previous reports (65, 66), nonmuscle myosin II was localized primarily along stress fibers at the basal surfaces of COS7 cells (Fig. 10, b, e, h, k, and n). The supervillin distribution partially overlapped that of myosin II, especially along stress fibers, but much more supervillin than myosin was observed within surface membrane extensions (Fig. 10, d–f). The localization of myosin II was not disrupted by expression of moderately high levels of EGFP (Fig. 10, a–c), moderately high levels of EGFP-tagged full-length supervillin (Fig. 10, d–f), or low levels of EGFP-SV-(1–174) (Fig. 10, g–i). By contrast, moderately high levels of EGFP-tagged SV-(1–174) induced a re-distribution of the nonmuscle myosin II staining into dot-like structures in 32.7 ± 8.9% (S.E., n = 3, p < 0.01) of the transfected cells (Fig. 10, j–l). In these cells, many fewer basal microfilaments stained for myosin II, and of those that did, staining was much less uniform along their length (Fig. 10k). Normal distributions of myosin II were observed in cells expressing moderately high levels of SV-(1–134) (Fig. 10, m–o), suggesting that the overexpression phenotype of SV-(1–174) is a dominant-negative effect caused by increased levels of the supervillin myosin-binding site divorced from the other cytoskeleton-associated sequences in this protein. These results show that EGFP-SV-(1–174) can induce a redistribution of myosin filaments in vivo and support the in vitro demonstrations of interactions between myosin II and the supervillin N terminus.

**DISCUSSION**

In this study, we have identified four sequences within the supervillin N terminus that mediate associations with the actin-based cytoskeleton. Three of these supervillin sequences mediate direct binding to actin filaments, and a fourth binds directly to myosin II (Fig. 11). Binding of all four supervillin sequences has been demonstrated with purified proteins (Figs. 2, 7, and 9) and by co-localization and reorganization of target cytoskeletal structures in vivo (Figs. 5 and 10).

Minimal sequences required for the binding to myosin II are supervillin amino acids 11–174 and sequences within nonmuscle myosin S2 domains, and three F-actin-binding sites (blue), located within supervillin amino acids 291–342 (A1), 340–450 (A2), and 748–805 (A3), respectively, exhibit the potential to mediate actin filament organization. Taken together, these interactions suggest that supervillin may contribute to the organization of actin and myosin filaments at the cytoplasmic surface of the plasma membrane.
subdomain S2. Binding of SV-(1–174) to S2 in avian smooth muscle myosin II is approximately stoichiometric and of sufficiently high avidity to resist extensive washing (Figs. 6–9A). Interactions between SV-(11–174) and S2 sequences from human nonmuscle myosin II A were also identified in an untargeted yeast two-hybrid assay (Fig. 9, B and C). Furthermore, overexpression of SV-(1–174) induced reorganization of filamentosous nonmuscle myosin II in vivo (Fig. 10k). Because supervillin N-terminal sequences did not bind significantly to rabbit skeletal muscle myosin II (Fig. T, D and E), binding may be selective for nonmuscle and smooth muscle myosins. In agreement with this idea, the region of myosin S2 that binds SV-(1–174) contains even more amino acids that are identical between smooth muscle and nonmuscle myosins but divergent in skeletal muscle myosin (Fig. 11A, yellow shading) than residues that are conserved in all three types of myosin II (Fig. 11A, red shading).

The minimal supervillin S2 sequence required for binding to supervillin may lie between smooth muscle myosin Lys-887 and Asn-1030 (Fig. 11A). The smallest myosin sequence identified as a strong positive in the yeast two-hybrid screen corresponds to chick smooth muscle myosin residues 887–1109 (Fig. 11A). The prominent binding of 125I-SV-(1–174) to HMM2 in blot overlays (Fig. 8B, lane 5) indicates that sequences N-terminal to Asn-1030 are sufficient for binding, albeit at a possibly reduced affinity. The greatly diminished binding to rod in blot overlays (Fig. 8B, lane 3) supports the importance of residues at or near the S1-S2 junction (amino acids 849–946 (67)), a region of smooth muscle myosin II that has been suggested to play an important role in the regulation of myosin activity and assembly, both directly (46, 68, 69) and through binding of regulatory proteins (70–74).

Supervillin sequences capable of binding to actin filaments include a site near the C terminus of SV-(171–342), the N terminus of SV-(343–571), and the C terminus of SV-(570–830) (Fig. 2C). Each of these three F-actin-binding sites appears to bind along the sides of actin filaments. First, direct binding to 125I-labeled F-actin, nucleated and capped by plasma gelsolin and stabilized by phallolidin, has been demonstrated for each of the three supervillin fragments (Fig. 2B). This assay has been shown previously to be selective for proteins that bind to the sides, rather than to the ends of actin filaments (61). Binding of 125I-labeled F-actin to full-length supervillin is completely competed by excess amounts of myosin S1 in the absence, but not in the presence, of MgATP but is not affected by barbed-end filament capping proteins (29). Multiple lateral associations with actin filaments also are indicated by the increased affinities for sedimented F-actin (Fig. 2A) and the actin filament bundling and cross-linking activities (Figs. 3 and 4) that we have observed for supervillin fragments containing at least two F-actin-binding sites. Thus, the in vivo F-actin binding and bundling activities of the supervillin N terminus (Fig. 5) (31) can be fully explained by the three F-actin-binding sites identified here.

The regions of the supervillin N terminus that contain the actin- and myosin-binding sites have been especially well conserved during evolution. Alignment of bovine, human, murine, and rat supervillin sequences identify six regions with especially high proportions of amino acids that are identical in all four supervillins (Fig. 11B). These highly conserved regions correspond to amino acids 1–60, 93–136, 291–319, 383–444, 695–723, and –746–809 in the bovine supervillin sequence. Of these, one or both of the first two conserved regions may be involved in binding to myosin S2 (Fig. 11B, Myosin), although residues 1–10 are not required for this interaction (Fig. 9B). The third, fourth, and sixth conserved regions fall within sequences that contain binding sites for F-actin (Fig. 11B, F-actin). We have estimated these binding sites to lie within residues 291–342, 343–450, and 748–830, based on the molecular masses of C-terminally truncated supervillin fragments that do and do not co-sediment with F-actin (Fig. 2C). These correlations are consistent with the presence of highly conserved binding sites for myosin S2 and F-actin in the supervillin N terminus.

Other functional binding domains also may be present within the supervillin N terminus. For instance, filamin binds directly or indirectly to SV-(1–174) at an unknown site (Fig. 6A). In addition, the loss of basal microfilament bundles induced by overexpression of SV-(343–571) (Fig. 5, s→u) is unlikely to be caused by a single actin-binding site. Although we cannot exclude the possibility that an additional binding site for F-actin is present in SV-(343–571), if this fragment does contain more than one binding site for F-actin, they each must be of relatively low avidity. Similar amounts of co-sedimentation with F-actin were observed for SV-(343–571) as for SV-(171–342) and SV-(570–830) (Fig. 2A), each of which contains a single C-terminal actin-binding site (Fig. 2C). Finally, the existence of an N-terminal membrane attachment site is suggested by the membrane association of SV-(1–380) in vivo (31) and by the continued binding of supervillin to cholesterol-rich membrane domains stripped of detectable actin by extraction with sodium carbonate (25). Interestingly, significant amounts of nonmuscle myosin II co-associate with supervillin and intrinsinc membrane proteins under these conditions, suggesting the possibility that supervillin may mediate an actin-independent linkage of myosin II to the membrane.

Supervillin also may mediate the interaction between myosin II and actin filaments (Fig. 11C). Such a function is well accepted for other proteins known to bind to both myosin and actin filaments. These proteins include myosin light chain kinase, calponin, and caldesmon (70, 71, 73–75). Although calponin and caldesmon have been investigated primarily in smooth muscle, nonmuscle isoforms for all three of these proteins exist. Thus, one hypothesis for the myosin II redistribution induced by overexpressed SV-(1–174) (Fig. 10, j→i) is a dominant-negative effect caused by competition with other myosin S2-binding proteins. If true, then the normal appearing distribution of myosin II in the presence of overexpressed SV-(1–342) (Fig. 10, m→o), a fragment that contains an actin-binding site as well as the myosin-binding site, implies that supervillin may organize actin and myosin filament interactions in ways similar to those proposed for caldesmon and calponin. The tight association of supervillin with liquid-ordered membrane domains (25) and its localization at actin-rich membrane punctae (25, 31) further suggest that supervillin is well suited to promote the recruitment to, and/or to regulate the interaction between, actin and myosin filaments at these regions of the membrane. As the only protein known so far to be capable of linking liquid-ordered membrane domains to both F-actin and myosin II, supervillin may be an important "adapter" protein for the organization of membrane skeleton attachments at these dynamic regions of the plasma membrane.

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Yu Chen, Norio Takizawa, Jessica L. Crowley, Sang W. Oh, Cheryl L. Gatto, Taketoshi Kambara, Osamu Sato, Xiang-dong Li, Mitsuo Ikebe and Elizabeth J. Luna

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