Localization by Segmental Deletion Analysis and Functional Characterization of a Third Actin-binding Site in Domain 5 of Scinderin*

Monica G. Marcu, Li Zhang, Abdelbaset Elzagallaai, and José-Maria Trifaro‡

From the Secretory Process Research Program, Department of Pharmacology, Faculty of Medicine, University of Ottawa, Ottawa, Ontario K1H 8M5, Canada

Scinderin is a Ca\(^{2+}\)-dependent actin filament severing protein present in a variety of secretory cells. Previous work suggests that scinderin-evoked cortical F-actin disassembly is required for secretion because local disassembly of cortical cytoskeleton allows secretory vesicle exocytosis (Vitale, M. L., Rodriguez Del Castillo, A., Tchakarov, L., and Trifaro, J.-M. (1991) *J. Cell Biol.* 113, 1057–1067). Scinderin has six domains each containing three internal sequence motifs, two actin, and two phosphatidylinositol disphosphate-binding sites in domains 1 and 2. In this paper we report the presence of another actin-binding site at the NH\(_2\)-terminal of domain 5 (Sc\(_{511–518}\)). This site binds actin in a Ca\(^{2+}\)-independent manner and a recombinant fragment (Sc\(_{5–6}\) or Sc\(_{502–715}\)) containing this site binds to actin-DNase-I-Sepharose 4B beads, co-sediments with actin and is able to nucleate actin assembly. Recombinant Sc\(_{5–6}\) and Sc-ABP3 also prevented the actin severing activity of intact scinderin. Moreover, Sc-ABP3, a peptide constructed with sequence (RLFQVRRNLASIT) identical to Sc\(_{511–523}\) blocked the binding of Sc\(_{5–6}\) to actin. Sc\(_{5–6}\) and Sc-ABP3 also prevented the actin severing activity of recombinant full-length scinderin (r-Sc) and inhibited the potentiation by r-Sc of Ca\(^{2+}\)-evoked release of serotonin from permeabilized platelets. On the other hand, Sc\(_{5–6}\) failed to block the effect of r-Sc on platelet serotonin release. Sc\(_{1–4,6}\), a construct devoid of domain 5, was able to sever but unable to nucleate actin, indicating that an actin nucleation site of scinderin was in domain 5. The results suggest that scinderin, in addition to binding actin on sites present in domains 1 and 2, must bind actin on a third site in domain 5 to sever and nucleate actin effectively.

Trifaró *et al.* (1), Bader *et al.* (2), and Ashino *et al.* (3) first showed the presence of gelsolin-like proteins in chromaffin cells. Bader *et al.* (2) also showed the presence of a Ca\(^{2+}\)-dependent actin-binding protein that was immunologically different from gelsolin. The isolation and characterization of this protein was carried out simultaneously by two independent laboratories (4–6). These publications showed that chromaffin cells contain a Ca\(^{2+}\)-dependent actin-severing protein that was different from gelsolin, although gelsolin was also present in these cells (2, 5). The names “scinderin” (4, 5) and “adseverin” (6) were given to this protein. Cloning of scinderin cDNA and sequence analysis (7, 8) demonstrated that, similarly to gelsolin, scinderin has six domains with two actin-binding sites in domains 1 and 2 (7, 9, 10). Two Ca\(^{2+}\)-binding sites were also described for scinderin (5), and contrary to gelsolin (11–13) but similar to villin (14, 15), scinderin dissociated from actin in the presence of EGTA (4–6). Similar to gelsolin and villin, the actin-severing activity of scinderin resides in its NH\(_2\)-terminal half (16), precisely domains 1 and 2 (7, 9, 10). The F-actin severing activity of scinderin seems to play a role in secretion (17). Scinderin is present only in secretory cells (5, 18, 19) and in platelets as well as in chromaffin cells is highly concentrated together with F-actin in the cortex (17). Cortical F-actin acts as a barrier to the free movement of secretory vesicles to release sites, and only during cell stimulation induced Ca\(^{2+}\) entry, cortical F-actin is disassembled, most probably through the activation of scinderin (17). This will allow the movement of secretory vesicles to release sites on the plasma membrane (17, 20). In this regard, recent experiments have shown that recombinant scinderin potentiates Ca\(^{2+}\)-evoked F-actin disassembly and exocytosis in platelets as well as in chromaffin cells (9, 10), effects blocked by PIP\(_2\) (9, 10). Two PIP\(_2\)-binding sites have been described for scinderin, and as with gelsolin, they are present in segments 1 and 2 (7). PIP\(_2\) inhibits the actin-severing activity of scinderin, but unexpectedly phosphatidylinerine also binds to scinderin and inhibits its actin-severing activity (21, 22). This is a distinct feature of scinderin when compared with gelsolin. Although the actin-severing activity of scinderin is localized in its NH\(_2\)-terminal half (16), and this binds a molecule of G-actin (20), the COOH-terminal half also binds a molecule of G-actin (16, 20) with formation of a 2:1 scinderin-actin complex (20).

The present experiments were directed to identify and characterize a third actin-binding site that was found to be present in the COOH-terminal half of scinderin. Fusion proteins corresponding to either the full-length or truncations of scinderin were prepared and tested for their interaction with actin. Here we demonstrate that a third binding site for actin is localized in the NH\(_2\)-terminal half of domain 5 of scinderin. Moreover, experiments with a recombinant truncated scinderin containing this site or a peptide constructed on the basis of this site sequence demonstrated that when this third site is occupied or blocked, the severing activity of intact scinderin is reduced. Similarly, either this peptide or a truncated scinderin fragment containing domain 5 was able to block the increases in Ca\(^{2+}\)-evoked release of serotonin from platelets induced by recombinant full-length scinderin, suggesting again that the third ac-

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‡ To whom correspondence should be addressed: Dept. of Pharmacology, University of Ottawa, 451 Smyth Rd., Ottawa, ON K1H 8M5, Canada.

1 The abbreviations used are: PIP\(_2\), phosphatidylinositol disphosphate; r-Sc, recombinant full-length scinderin; TRX, thioredoxin; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; PIPES, 1,4-piperazinediethanesulfonic acid; 5-HT, 5-hydroxytriptamine.
tin-binding site is necessary for full interaction of scinderin with actin. Furthermore, experiments with a scinderin construct with deletion of domain 5 indicated that the actin-binding site present in this domain contributes to the actin nucleation activity of scinderin.

MATERIALS AND METHODS

Protein Expression and Purification—The Thioredoxin (TRX) Thio-Fusion System (Invitrogen, San Diego, CA) was used for the expression of fusion proteins. Full-length recombinant scinderin (r-Sc) was prepared as described previously (9, 10), and the different truncations (deletions) of scinderin were obtained by PCR. Primers were designed to include a BamHI restriction site for 5’ end and a SaI 3’ end. The obtained PCR products were digested and subcloned in expression vector pTrxFus using the same restriction sites. Recombinant plasmids were then transfected into the host Echerichia coli GI698. Correct subcloning and reading frames of all clones were confirmed by dideoxy sequencing (ABI 373 Automated Sequencer). PCR primers used to construct the recombinant proteins were as follows: 5’-ACT GCA GGA TCC ATT GGT TTC AGA TGC CAG TGG-3’ forward for Sc3–6, Sc3–4, and Sc3–1⁄24; 5’-GTC GTG TCG ACA GTA GTC AGC ATC TGG TA-3’ reverse for Sc, Sc5–6, Sc5–6, Sc1⁄25–6, and Sc6; 5’-GTC GTG TCG ACT TGC TCG TTC CCA GGT GTA G-3’ reverse for Sc3–4; 5’-ACT GCA GGA TCC AGA GAA AGG AGC AGA GTA CG-3’ forward for Sc1–4,6; 5’-ACT GCA GGA TCC AGC ATC CAG CAG CAG AGC TA-3’ reverse for Sc1–4,6; and 5’-ACT GCA GGA TCC AGC ATC CAG CAG CAG AGC TA-3’ reverse for Sc1–4,6.

GGA TCC AGA AGG TCA GGC ACC AGC C-3

GCA GGA TCC AGC ATC CAG CAG CAG CAG AGC TA-3

GenBank HI restriction site for 5’ BamHI and 3’ HindIII. One new amino acid was introduced just in front of the amino acid sequence of segment 6 of scinderin. Transformed E. coli were grown in Rich Medium containing 100 μg ampicillin/ml and induced with 100 μg tryptophan/ml. Proteins were obtained by osmotic shock to a purity of at least 80%, as described previously (9). For the release studies, r-Sc was further purified by actin-DNase-I-Sepharose 4B affinity chromatography as described by Marcu et al. (9). Fusion proteins were dialyzed against deionized water and lyophilized as described previously (9, 10).

A schematic representation of the position of the different recombinant fragments in the scinderin molecule is shown in Fig. 1.

**Actin Binding Assay**—Actin-DNase-I-Sepharose 4B affinity beads were prepared as described previously (9) and used in binding experiments performed “in bulk.” Rabbit skeletal muscle actin (G-150) was purchased from Cytoskeleton (Denver, CO). The buffer used for binding assays was either 1 mM CaCl2, 150 mM KCl, 1 mM dithiothreitol, 1 mM NaATP, 10 mM Tris-HCl, pH 7.4, or the same buffer devoid of Ca2+ but containing 10 mM EGTA (Ca2+-free buffer). Aliquots of fusion proteins were mixed with actin and incubated for 90 min at 20 °C. Different ratios between recombinant truncated proteins and actin were used: 0.15:1; 0.35:1; 0.5:1; 0.75:1; 0.95:1; 1.1:1; and 1.2:1. Filamentous actin was sedimented by centrifugation at 100,000 × g for 60 min; sediments and supernatants were tested for the presence of proteins by SDS-PAGE and Western blots. To determine affinities to actin and Kc values of scinderin constructs, 4 μl actin samples were polymerized in the presence of different molar ratios of constructs to actin. After 90 min of incubation, the mixtures were centrifuged as above. The amounts of constructs and actin in supernatants and pellets were determined by densitometric scanning (Ultrascan XL Broma Laser densitometer) of Coomassie Blue-stained gels. Scatchard’s plots were prepared with the data, and Kc values were calculated from the plots.

**Viscometry Analysis**—Apparent viscosity was measured at low shear rates by the falling ball technique as described by MacLean-Fletcher and Pollard (24). G-actin was induced to polymerize (4 mg/ml) for 1 h at 22 °C, and then it was diluted to 1 mg/ml (0.22 μM) in the following buffer: 100 mM KCl, 2 mM MgCl2, 5 mM EGTA, 0.005% NaN3, 10 μM free...
Fig. 3. Binding of recombinant scinderin fragments to actin-DNasel-Sepharose 4B beads. A, truncated scinderins were tested for binding to actin in the presence of 1 mM Ca$^{2+}$ as indicated under “Materials and Methods.” Because two of the bound proteins (Sc3–6 and Sc5–6) could not be eluted with either buffer A or B (Ca$^{2+}$-free 10 mM EGTA), at the end of incubations, the Sepharose 4B beads carrying the protein complexes were boiled with the SDS sample buffer, and the supernatants were loaded onto the gel. Therefore, actin (*) and DNase I (△) are also visible in the Coomassie Blue-stained gel. Only recombinants Sc3–6 and Sc5–6 were found to be bound to actin under these conditions. B, because domain 4 of gelsolin has been shown to contain an actin-binding site (36), an additional experiment was carried out to further analyze Sc3–4 lack of binding to actin using Sc5–6 as a positive control. In the absence of actin and the presence of either Sepharose 4B (lanes 1 and 2) or Sepharose 4B-DNase I (lanes 3 and 4), both Sc3–4 and Sc5–6 did not bind to the beads, and they were recovered in the supernatants (lanes 1′–4′). When Sepharose 4B-DNase I-actin beads were present in the medium (lanes 5 and 6), Sc3–4 did not bind, and it was recovered in the supernatant (lane 5′). On the other hand, Sc5–6 was found to be bound to the beads (lanes 6 and 6′). Actin and DNase I bands are indicated by * and △ respectively. C, Western blot of an experiment performed to test the binding of Sc3–6 and Sc6–6 in either 1 mM Ca$^{2+}$ or Ca$^{2+}$-free 10 mM EGTA. Both proteins bind well under these two conditions. Samples were prepared as above.

Ca$^{2+}$ (4.6 mM CaCl$_2$), 1 mM NaATP, 1 mM phenylmethylsulfonyl fluoride, 1 mg leupeptin/ml, 40 mM PIPES, pH 6.8. Actin alone or mixed (at 0 °C) with fusion proteins in different ratios was drawn into 100-μl glass capillaries, sealed with plasticine at one end, and held horizontally at room temperature for 2 h before determination of the apparent viscosity. One stainless steel ball was used per capillaries; one measurement per tube and three measurements per condition were done. Capillary tubes were placed at an angle of 45°, and the time for the ball to fall between two points was recorded. Apparent viscosity was calculated from a calibration curve obtained by measuring ball falling times through glycerol solutions (from 0–100%) of known viscosity, and the results were expressed in centipoises.

Actin Nucleation Assay—6 μM G-actin containing 5% pyrene-iodoacetamide-labeled actin (Cytoskeleton, Denver, CO) were dissolved in low salt buffer (0.2 mM NaATP, 0.5 mM dithiothreitol, 0.2 mM CaCl$_2$, and 5 mM Tris-HCl, pH 8.0) and left on ice for 30 min to allow complete depolymerization of small oligomers that might form during defrosting. The solution was then centrifuged at 100,000 × g for 1 h at 4 °C, and the solution present in the supernatant was polymerized alone or in the presence of r-Sc or recombinants Sc1–4, Sc5–6, or ScL5–6 (the molar ratio of actin to any of the recombinants was 400:1) by addition of 1/50th volume of polymerization inducer buffer (2.5 M KCl, 100 mM MgCl$_2$, and 50 mM NaATP). Actin polymerization was monitored for 120 min at 22 °C by measuring the change in fluorescence, using a 10-nm bandpass and excitation and emission wavelengths of 365 and 407 nm, respectively (Perkin-Elmer LS5 Fluorometer).

Gel Electrophoresis and Immunoblotting—Monodimensional electrophoresis (SDS-PAGE) was performed according to Doucet and Trifaro (25). Gels were either stained with Coomassie Blue or transferred to nitrocellulose membranes. Immunolabeling was performed with a monoclonal antibody against thioredoxin (Invitrogen, CA) in 1:5000 dilution. The solution was then centrifuged at 100,000 × g for 1 h at 4 °C, and the solution present in the supernatant was polymerized alone or in the presence of r-Sc or recombinants Sc1–4, Sc5–6, or ScL5–6 (the molar ratio of actin to any of the recombinants was 400:1) by addition of 1/50th volume of polymerization inducer buffer (2.5 M KCl, 100 mM MgCl$_2$, and 50 mM NaATP). Actin polymerization was monitored for 120 min at 22 °C by measuring the change in fluorescence, using a 10-nm bandwidth and excitation and emission wavelengths of 365 and 407 nm, respectively (Perkin-Elmer LS5 Fluorometer).

Preparation of Scinderin-derived Peptides—Peptides with a sequence corresponding to the 5′ end of scinderin 5–th domain (RLFQVRRNLASIT) and with scramble sequence (AVNIRLRFSTLQR) were prepared by solid phase peptide synthesis (26). Sequences were checked by Ed-
Platelet Permeabilization, Labeling of Serotonin Store, and Serotonin Release—Platelet-rich plasma was obtained from the Ottawa Red Cross and centrifuged at 200 × g for 15 min to obtain a platelet sediment. This sediment was resuspended in Ca2+-free Locke’s solution (154 mM NaCl, 2.6 mM KCl, 2.14 mM KH2PO4, 0.85 mM KH2PO4, 1.2 mM MgCl2, 10 mM glucose, and 2.0 mM EGTA, pH 7.2), and the platelet concentration was adjusted to 7.5 × 10^9/ml. Platelets were then incubated at 37°C for 90 min with 0.6 nmol [3H]5-hydroxytryptamine (serotonin, [H][HT]ym) (specific activity = 55.4 Ci/mmol, DuPont) (28), and [H][HT]-labeled platelets were washed by incubation with six changes of 10 ml of Ca2+-free Locke’s solution over a 60-min period. [H][HT]-labeled platelets were permeabilized by 5 min of treatment with 15 μM digitonin in K-glutamate buffer (160 mM K-glutamate, 12.5 mM MgCl2, 2.5 mM EGTA, 2.5 mM EDTA, 5 mM ATP, 20 mM HEPES, pH 7.4) (29). Platelets were then centrifuged at 900 × g for 2 min (4°C) and resuspended in K-glutamate buffer. Ca2+ concentrations (pCa values) were calculated as described previously (30). Samples (100 μl) containing 7.5 × 10^9 permeabilized platelets in K-glutamate buffer were stimulated with 10 μM Ca2+ for 45 s in the absence or the presence of different recombinant scinderins, a scinderin-derived actin-binding peptide, or both. Release experiments were carried out as described previously (9). [H][HT] output was expressed as a percentage of total content after subtraction of values for spontaneous (absence of Ca2+) release. A minimum of eight samples/condition were measured, and the means ± S.E. were plotted.

RESULTS

Recombinant Scinderins: Expression and Purification—Different truncations of scinderin cDNA obtained by PCR were subcloned into expression vector pTrxFus using the restriction sites as indicated under “Materials and Methods.” Recombinant vectors were introduced into E. coli GI698 to express the corresponding fusion proteins. Lysates obtained from tryptophan-induced cultures showed in SDS-PAGE a new band that corresponded to each truncated scinderin (see Fig. 2). The molecular masses of these TRX fusion proteins were as follows: Sc1–4, 69 kDa; Sc3–4, 64 kDa; Sc5–6, 43 kDa; Sc3–4, 34 kDa; Sc5–6, 41 kDa; Sc5–6, 40.6 kDa; Sc1–5–6, 35 kDa; and Sc5, 27 kDa. A schematic representation of the position of these scinderin fragments in the scinderin molecule is shown in Fig. 1. Some fusion proteins (Sc1–4,6 and Sc3–4 to Sc5–6) were extremely well expressed amounting to more than 50% among total E. coli proteins (Fig. 2, A and C). Because the scinderin polyclonal antibody did not recognize several scinderin fragments (only Sc1–4,6, Sc3–4, and Sc3–4 were recognized by the antibody), most Western blots (Fig. 2B) were stained with a mouse TRX monoclonal antibody (Invitrogen, CA). However, when dealing with r-Sc (Sc1–6) and Sc1–4,6 blots were stained with a scinderin polyclonal antibody (Fig. 2D). Enterokinase does not efficiently cut TRX-Sc fusion proteins, and because scinderin is very sensitive and undergoes fast proteolysis at room temperature, all experiments were carried on with TRX-Sc fusion proteins as previously shown (9, 10). TRX does not interfere with the function of scinderin (9, 10) and has no effect in vitro tests when compared with TRX-Sc (data not shown).

Binding of Recombinant Scinderin Fragments to Actin—The ability of scinderin fragments to bind actin was tested using two different assays. (a) The first assay was binding to actin-DNaseI-Sepharose 4B beads. In Ca2+ containing buffer (buffer A), only two Sc-derived proteins, Sc3–6 and Sc5–6, were found to bind actin (Fig. 3A). Moreover, once bound in the presence of 1 mM Ca2+, Sc3–6 and Sc5–6 could not be eluted by a EGTA buffer (buffer B). As a matter of fact, both Sc3–6 and Sc5–6 were found to bind to actin equally well in the presence or absence (10 mM EGTA) of 1 mM Ca2+ (Fig. 3C). Due to the fact that the Sc3–6 and Sc5–6 could not be eluted with EGTA containing buffer, all gel wells were loaded with supernatants obtained after the Sepharose 4B beads were boiled in SDS-PAGE sample buffer. Therefore, the presence of actin and DNase I was evident in all Coomassie Blue-stained gels (Fig. 3, A and B). A deletion of 16 amino acids (502–518) from the beginning of domain 5 produced Sc5–6, a fragment corresponding to amino acids 519–715 of scinderin, which did not bind actin. This suggests the presence of an actin-binding site at NH2-terminal of domain 5. Because it has been shown that domain 4 of gelsolin contains an actin-binding site (36), a set of experiments tested Sc3–4 and Sc5–6 for binding under different conditions (Fig. 3B). These two constructs did not bind to either Sepharose 4B or Sepharose 4B-DNase I beads (Fig. 3B, lanes 1–4). When tested for binding to Sepharose 4B-DNase I-actin beads, only Sc5–6 was found to bind to the beads (Fig. 3B, lane 6). (b) The second assay was co-sedimentation of recombinant truncated scinderins with actin. Here again, with the exception of Sc3–6 (data not shown) and Sc5–6, all recombinant scinderin fragments failed to co-sediment with actin under these experimental conditions (Fig. 4A). This, together with the observation that Sc5–6 did not co-sediment with actin, whereas Sc6–5 did, suggests the presence of a third actin-binding site at the 5′ end of domain 5 (Sc5) of scinderin. To further demonstrate the presence of a third actin-binding site a 13-amino acid peptide (Sc-ABP3) with the sequence RLFQVRNLASIT was constructed. This peptide is 6 amino acids longer than the possible actin-binding site (RLFQVRN). The length of the peptide was chosen just to be sure that the
difference in binding to actin between Sc5–6 and Sc L5–6 was not due to a possible disruption of a binding site whose length remains, at this point, to be elucidated. The scinderin-derived peptide (Sc511–523 or Sc-ABP3) but not a scrambled 13-amino acid peptide (AVNIRLRFSTLQR; no sequence homology in the EBI Data Bank), when present in a ratio to actin of 10:1, reduced by 49% (as shown by densitometry measurements) the binding of either Sc5–6 or Sc3–6 (data not shown) to actin when tested in the actin co-sedimentation assay (Fig. 4B). The binding of Sc5–6 to actin in the presence of the scramble peptide was 101% of that observed in its absence. The deletion of 16 amino acids that produced ScL5–6 was enough to suppress the binding to actin. Consequently, the actin-binding site should be localized at the very beginning of domain 5 of scinderin. This is a stretch of 13 amino acids starting at amino acid 511 and ending at amino acid 523.

Affinity of the Sc5–6 Site for Actin—Actin co-sedimentation experiments were also performed at different construct:actin molar ratios to determine affinity constants \( (K_d) \). The experiments were carried out either in the presence or the absence (10 mM EGTA) of Ca\(^{2+}\) to determine whether or not Ca\(^{2+}\) would affect the affinity for actin. In these experiments, Sc3–4 was also included to rule out the possibility that the absence of its binding to actin described above was due to the fact that only one concentration of Sc3–4 and only one ratio to actin was tested. Again, the experiments clearly demonstrated that only Sc5–6 was bound to actin at all ratios tested (Fig. 5, bottom). Scatchard analysis of the data (Fig. 5, top) revealed a \( K_d \) for Sc5–6 of 0.30 \( \mu \)M when tested in the presence of Ca\(^{2+}\) and a \( K_d \) of 0.33 \( \mu \)M in Ca\(^{2+}\)-free medium; thus, indicating that Sc5–6 binds actin with equal affinity either in the presence or the absence of Ca\(^{2+}\).

Severing Activity of Recombinant Full-length Scinderin and Its Fragments—The severing activity of recombinant scind-
ABP3 was ineffective in blocking the actin severing activity of reducing the apparent viscosity of actin, and in this case, Sc-binding or activity of r-Sc, Sc1–4,6, a construct with a deletion peptide with the binding sequence) interferes either with the binding to actin by either domain 5 or Sc-ABP3 (a peptide with the binding sequence). Moreover, when r-Sc and Sc1–4,6 were tested in the presence of 10 μM Sc5–6 do not seem to have any effect on viscosity of actin gels (Fig. 6A). However, and as expected, r-Sc was very effective in decreasing viscosity (Fig. 6B). However, in the presence of either Sc5–6 or Sc-ABP3 in different molar ratios were tested in the presence of 10 μM Ca2+. Under these conditions r-Sc alone decreases the viscosity of actin gels (Fig. 6B). However, in the presence of either Sc5–6 or Sc3–6 (data not shown), r-Sc ability to decrease viscosity of actin gels was reduced (Fig. 6B). Moreover, when r-Sc was tested in the presence of Sc-ABP3, a similar inhibition of r-Sc effect on actin gels was observed (Fig. 6B). In view of these results, which indicate that the binding to actin by either domain 5 or Sc-ABP3 (a peptide with the binding sequence) interferes either with the binding or activity of r-Sc, Sc1–4,6, a construct with a deletion of domain 5, was tested. Sc1–4,6 was as effective as r-Sc in reducing the apparent viscosity of actin, and in this case, Sc-ABP3 was ineffective in blocking the actin severing activity of the construct (Fig. 6C). Moreover, when r-Sc and Sc1–4,6 were combined, there was a summation of their actin severing activities (Fig. 6C).

**Nucleation**—Actin polymerization was evaluated by measuring the increase in fluorescence of pyrene-labeled actin as indicated under “Materials and Methods.” In the presence of either r-Sc or Sc5–6 and after a lag period of approximately 10 and 15 s, respectively, the fluorescence intensity increased exponentially reaching a maximum at 110 s (Fig. 7). In the absence of r-Sc or Sc5–6, actin polymerized slowly reaching after 30 s 33% and after 120 min 30% of the fluorescence intensity reached in the presence of either recombinant scinderin, thus suggesting that spontaneous nucleation was very slow. Sc5–6 and Sc1–4,6 did not nucleate actin assembly (Fig. 7). The results indicate that Sc5–6, even when tested at a small molar ratio to actin (1:400) is a very powerful inducer of actin polymerization and that this activity is localized at the NH2-terminal end of domain 5 (Sc502–518).

**Effect of Sc5–6, Sc1–4,6, and Sc1–4,6 on the Increase in**

Sc1–4,6, and Sc-ABP3 showed that Sc3–6 (data not shown) and Sc5–6 do not seem to have any effect on viscosity of actin gels (Fig. 6A). However, and as expected, r-Sc was very effective in decreasing viscosity (Fig. 6A). Mixtures of r-Sc with actin in the presence of either Sc5–6 or Sc-ABP3 in different molar ratios were tested in the presence of 10 μM Ca2+. Under these conditions r-Sc alone decreases the viscosity of actin gels (Fig. 6B). However, in the presence of either Sc5–6 or Sc3–6 (data not shown), r-Sc ability to decrease viscosity of actin gels was reduced (Fig. 6B). Moreover, when r-Sc was tested in the presence of Sc-ABP3, a similar inhibition of r-Sc effect on actin gels was observed (Fig. 6B). In view of these results, which indicate that the binding to actin by either domain 5 or Sc-ABP3 (a peptide with the binding sequence) interferes either with the binding or activity of r-Sc, Sc1–4,6, a construct with a deletion of domain 5, was tested. Sc1–4,6 was as effective as r-Sc in reducing the apparent viscosity of actin, and in this case, Sc-ABP3 was ineffective in blocking the actin severing activity of the construct (Fig. 6C). Moreover, when r-Sc and Sc1–4,6 were combined, there was a summation of their actin severing activities (Fig. 6C).

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**Effect of Sc5–6, Sc1–4,6, and Sc1–4,6 on the Increase in**

Scinderin is a Ca2+-dependent actin-severing protein of 715 amino acids that shares 63 and 53% homology, respectively, with gelsolin and villin (7). Scinderin shares six internal repeats of short sequence motifs (A, B, and C) with gelsolin and
Scinderin Third Actin-binding Site

villin (7, 31). After alignment of motifs A, B, and C, these proteins reveal six (Sc1–6) domains (31). Previous work has demonstrated the presence and functional properties of two actin-binding sequences in domains 1 and 2 of scinderin (7, 9, 10). The high homology of these two actin-binding sites in these proteins (7, 31) would suggest that the type of interaction described for domains 1 and 2 of gelsolin with actin (32) might be similar for scinderin and villin. However, the binding of domain 1 of gelsolin to actin seems to be Ca²⁺-independent (33, 34), whereas in the case of scinderin, this binding requires the presence of Ca²⁺ (20). Therefore, because of this property, scinderin resembles villin rather than gelsolin.

The present experiments describe a third actin-binding site in scinderin. This site is localized to the NH₂-terminal half of segment 5 of scinderin because construct Sc5–6 but not Sc5–6 was able to bind actin. The first 5 amino acids in the sequence (RLFQVRNL) of this site (scinderin 511–519) are the same as those present in positions 161–165 of the second actin-binding site (RLFQVKGR) of gelsolin segment 2. The site (amino acids 511–519) present in Sc5–6 is able to bind actin monomers, similar to gelsolin S4–6 (35). However, the actin-binding site in this part of gelsolin has been localized to segment 4 (36). The results from different types of experiments (Figs. 3–5) presented here clearly indicate that segment 4 of scinderin has no actin binding properties. Furthermore, and opposite to what has been described for gelsolin S4–6 and similar to gelsolin S-2 (35), the binding of actin to Sc5–6 is Ca²⁺-independent, because the present experiments show that Sc5–6 was bound to actin DNase-I-Sepharose 4B beads or co-sedimented with actin quite effectively in the presence of 10 mM EGTA. Moreover, the affinity of Sc5–6 for actin was similar in Ca²⁺ or Ca²⁺-free solutions as indicated by similar Kᵣ values (0.30 and 0.33 μM) obtained by Scatchard plot analysis of the data obtained in these two buffer solutions. These Kᵣ values are within the range of those published for gelsolin domains S₁ (36) and S₂₋₃ (37). We have demonstrated that intact native scinderin interacts with actin in a Ca²⁺-dependent manner and is eluted from actin Sepharose 4B with EGTA buffers (4, 7). Therefore, it is quite possible that the third actin-binding site of scinderin is hidden and that it is only exposed upon binding of Ca²⁺ and/or actin to other scinderin sites with the consequent changes in the configuration of the protein. The binding of actin to Sc3–6 or Sc5–6 observed in a Ca²⁺-evoked release of serotonin. Furthermore, Sc1–4,6, in addition to increase Ca²⁺-evoked release of serotonin, was able to show a summation of effects when combined with r-Sc. All this suggests that the third actin-binding site of scinderin should be occupied by actin for scinderin to display full activity. This idea gained support when the severing activity of scinderin was evaluated by viscometry of actin gels. The decrease in viscosity produced by r-Sc was completely blocked in the presence of either Sc5–6 or peptide Sc-ABP₁ with amino acid sequence corresponding to the third actin-binding site). The inhibitory effect of Sc-ABP₁ was observed at micromolar concentrations. These concentrations were necessary because the peptide binds to actin (the scinderin substrate), an abundant cellular protein.

In summary, the experiments presented here demonstrate the presence of the third actin-binding site, which needs to be occupied by actin to position scinderin in such a way as to allow its full severing activity. The experiments do not discard the possibility that in addition to the two actin-binding sites present at the NH₂-terminal half of scinderin, other actin-binding sites may also be present, because a systematic study of the NH₂-terminal half of gelsolin recently published indicated the presence of an additional actin-binding sequence at the COOH-terminal of segment 2 of gelsolin (38).

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Monica G. Marcu, Li Zhang, Abdelbaset Elzagallaai and José-Mari?a Trifaró

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