The Specific NH$_2$-terminal Sequence Ac-EEED of α-Smooth Muscle Actin Plays a Role in Polymerization In Vitro and In Vivo

Christine Chaponnier,* Marc Goethals,‡ Paul. A. Janmey,§ Françoise Gabbiani,* Giulio Gabbiani,* and Joël Vandekerckhove‡

*Department of Pathology, University of Geneva, CH-1211 Geneva 4, Switzerland; ‡Flanders Institute of Biotechnology, Laboratory of Physiological Chemistry, University of Ghent, Ghent, Belgium; and §Experimental Medicine Division, Brigham and Women's Hospital, Boston, Massachusetts 02115

Abstract. The blocking effect of the NH$_2$-terminal decapeptide of α-smooth muscle (SM) actin AcEEED-STALVC on the binding of the specific monoclonal antibody anti–αSM-1 (Skalli, O., P. Ropraz, A. Trzeviak, G. Benzonana, D. Gillessen, and G. Gabbiani. 1986. *J. Cell Biol.* 103:2787–2796) was compared with that of synthetic peptides modified by changing the acetyl group or by substituting an amino acid in positions 1 to 5. Using immunofluorescence and immunoblotting techniques, anti–αSM-1 binding was abolished by the native peptide and by peptides with a substitution in position 5, indicating that AcEEED is the epitope for anti–αSM-1. Incubation of anti–αSM-1 (or of its Fab fragment) with arterial SM actin increased polymerization in physiological salt conditions; the antibody binding did not hinder the incorporation of the actin antibody complex into the filaments. This action was not exerted on skeletal muscle actin.

After microinjection of the α-SM actin NH$_2$-terminal decapeptide or of the epitopic peptide into cultured aortic smooth muscle cells, double immunofluorescence for α-SM actin and total actin showed a selective disappearance of α-SM actin staining, detectable at ~30 min. When a control peptide (e.g. α-skeletal [SK] actin NH$_2$-terminal peptide) was microinjected, this was not seen. This effect is compatible with the possibility that the epitopic peptide traps a protein involved in α-SM actin polymerization during the dynamic filament turnover in stress fibers. Whatever the mechanism, this is the first evidence that the NH$_2$ terminus of an actin isoform plays a role in the regulation of polymerization in vitro and in vivo.

ACTIN isoforms are characterized by slightly different sequences particularly at their NH$_2$-terminal ends (Vandekerckhove and Weber, 1978; Vandekerckhove and Weber, 1979). They are produced by distinct genes (Ponte et al., 1983) and the sequences of each of them are perfectly conserved in higher vertebrates. In adult animals the two cytoplasmic isoforms (β and γ) are present in all cells, albeit in variable proportions, and the four muscular isoforms are characteristically expressed respectively in skeletal (α-skeletal) and cardiac (α-cardiac) muscles or in different smooth muscles (SM)[1], α-SM actin being abundant in vascular SM and γ-SM actin being abundant in parenchymal SM, especially in the gastro-intestinal tract (Vandekerckhove and Weber, 1981; Skalli et al., 1987). Another feature of actin isoforms is their sequential expres-

sion during development, particularly studied in skeletal and cardiac muscles (Ruzicka and Schwartz, 1988; Woodcock-Mitchell et al., 1988). α-SM actin has also been shown to appear ex novo in stress fibers of fibroblastic cells during pathological situations involving contractile phenomena such as wound healing and fibrocontractive diseases (Janmey and Chaponnier, 1995). Despite the phylogenetic conservation and the highly coordinated expression pattern, there is no definite evidence of actin isoform differential functions. On the contrary, it has been shown that skeletal actin microinjected in cultured fibroblastic cells incorporates readily into stress fibers (McKenna et al., 1985).

Due to the high homology among actin isoforms it has been difficult to raise specific antibodies. A few years ago an antibody selectively recognizing α-SM actin was produced (Skalli et al., 1986b). We report here that the epitope of this antibody is the NH$_2$-terminal sequence AcEEED. Moreover we show that the interaction of the antibody or its Fab fragment with aortic SM actin lowers the critical monomer concentration for polymerization and that microinjection of the epitopic peptide in cultured SM cells induces the se-

Address correspondence to Dr. G. Gabbiani, Dept. of Pathology, University of Geneva, CMU, 1 rue Michel-Servet, CH 1211 Geneva 4, Switzerland. Tel.: 4122 702 5743. Fax: 4122 702 5746.

_____
1. *Abbreviations used in this paper:* SK, skeletal; SM, smooth muscle.

lective disappearance of α-SM actin from stress fibers, suggesting that the epitope sequence unique to α-SM actin plays a role in polymerization.

Materials and Methods

Peptides

Peptides with the NH_2-terminal sequences of the six mammalian actin isoforms, extending until Cys-10, and α-SM related peptides with substitution at the blocking group or in positions 1, 2, 3, 4, or 5 were synthesized with a synthesizer (431A; Applied Biosystems Inc., Foster City, CA) on p-hydroxymethylphenoxymethyl copolystyrene 1% divinylbenzene resin using the Fmoc chemistry procedure and following the manufacturer's instructions. The peptides were acetylated or butyrylated while being attached to the resin in the side-chain protected form with acetic or butyric anhydride after removal of the last fluorenylmethyloxycarbonyl group.

After cleaving the protective groups and the peptide-resin conjugates, the peptides were precipitated with t-methyl-butylether and collected by centrifugation at 2,000 g. The residue was further dried for 2 h in a Speed-vac concentrator (Savant Instruments, Farmingdale, NY), then redissolved in H_2O and adjusted to pH 8.0 with 4 M NaOH. The peptides were desalted over a G-25 column (2 × 25 cm) in H_2O. The eluates were monitored by UV absorbance at 254 nm.

Peptides were coupled to BSA essentially as described by Green et al. (1982) and Gimona et al. (1994). 25 mg of BSA dissolved in 2 ml of 10 mM phosphate buffer, pH 7.2, were allowed to react with 4.2 mg of maleimidobenzoyl-N-hydroxysuccinimide dissolved in 50 μl dimethyl formamide under argon atmosphere. Excess reagent was then removed by gel-filtration in 50 mM phosphate buffer at pH 6.0 and the protein-containing fraction collected and adjusted with NaOH to pH 7.0. Each peptide (2 mg) was consecutively coupled for 3 h to 700-μl aliquots of the MBS-activated BSA (~1/20 of the amount collected after gel filtration). Aliquots were mixed with sample buffer for SDS–gel electrophoresis and Western blotting. The peptides AcEEED-NH_2 and EEED-NH_2 were purchased from Research Genetics (Huntsville, AL).

Protein Purification

Preparation of acetone powder of bovine aorta tunica media and purification of SM actin were carried out according to Strzelecka-Golaszewska et al. (1985). Actin from rabbit skeletal muscle acetone powder was purified by the method of Spudich and Watt (1971).

Anti-αSM-1 IgG2a (Skalli et al., 1986b) was purified from hybridoma supernatants by affinity chromatography on protein A–Sepharose 4B (Pharmacia, Uppsala, Sweden) and dialyzed against G-actin buffer (buffer G: 2 mM Tris-HCl, 0.2 mM ATP, 0.5 mM 2-mercaptoethanol, 0.2 mM $CaCl_2$, final pH 7.8; Spudich and Watt, 1971) for sedimentation assays.

A two-step method for the preparation of the monovalent Fab fragment from purified anti-αSM-1 was used because of the peculiar sensitivity to papain digestion of the IgG2a molecule (Parham, 1986). The $F(ab')_2$ was obtained by limited proteolytic digestion with pepsin at pH 4.2 (Parham, 1986). The intact immunoglobulin and the Fc portion were retained on a protein A–Sepharose column (Pharmacia). The flow through fraction, containing the $F(ab')_2$ portion, was dialyzed against 0.1 M Tris-HCl, pH 7.5, and reduced with a freshly made cysteine solution (10 mM final) at pH 7.5 for 2 h at 37°C to obtain the monovalent Fab fragment. Alkylation was performed using 0.15 M final iodoacetamide for 30 min at room temperature.

SDS-PAGE and Immunoblots

Electrophoresis of proteins was performed on 5–20% gradient SDS–polyacrylamide gels according to Laemmli (1970). Immunoblots were essentially performed according to Towbin et al. (1979) with minor modifications (Chaponnier et al., 1990). Anti-αSM-1 hybridoma supernatant was diluted 1:500 and peroxidase-conjugated affinity-purified goat–anti–mouse IgG plus IgM (Jackson ImmunoResearch Laboratories, West Grove, PA) 1:10,000. The membrane bound peroxidase-labeled antibodies were detected using the ECL Western blotting system (Amersham, Rahn AG, Zürich, Switzerland), according to the instructions of the manufacturer. For quantification, Coomassie blue stained gels and films were scanned with a computerized laser beam densitometer (Genofit, Geneva, Switzerland).

Sedimentation Assays

Stock solutions of 10 μM G-actin (aortic smooth muscle or skeletal muscle actin), 5 μM anti-αSM-1 IgG, 10 μM anti-αSM-1 Fab, buffer G, and Hisalt buffer (300 mM KCl, 6 mM $MgCl_2$ in buffer G) were used for polymerization assays. All proteins were dialyzed against buffer G. G-actin (50 μl) was mixed with: (a) buffer G (100 μl), (b) buffer G (50 μl) and Hisalt buffer (50 μl), (c) anti-αSM-1 IgG or Fab (50 μl) and Hisalt buffer (50 μl), (4) anti-αSM-1 IgG or Fab (50 μl) and buffer G (50 μl) and allowed to polymerize at room temperature for 30 min. F-actin and bound proteins were sedimented at 30 psi for 60 min in a Beckman Airfuge using an A-100 fixed-angle rotor (Beckman Instrument Co., Fullerton, CA). Equivalent amounts of the pellets and supernatants were analyzed by SDS-PAGE (Chaponnier et al., 1985).

Control experiments were performed to rule out nonspecific interactions. An unrelated IgG was used instead of anti-αSM-1; moreover excess peptides (decapeptides P1 and P2, tetrapeptides AcEEED and EEED) were mixed for 10 min at room temperature with anti-αSM-1 before the addition of actin for the 30 min polymerization time before centrifugation (see above).

Pyrene-Actin Assays

The rate and extent of actin polymerization were measured using pyrene-labeled α-SM and α-skeletal (SK) actins. Pyrene-labeled actin monomers undergo a characteristic 20-fold increase in fluorescence intensity when incorporated into filaments (Kouyama and Mihashi, 1981). The rate and extent of the fluorescence increase are directly related to the rate and extent of actin polymerization. α-SM actin was labeled by the method of Kouyama and Mihashi (1981). To measure the effects of anti-αSM-1 on polymerization, a concentration of labeled α-SM actin (α-SM PIA) or α-SK actin (α-SK PIA) below the critical concentration required for spontaneous polymerization was mixed with various concentrations of unlabeled α-SM or α-SK actin and anti-αSM-1. The rate of polymerization was determined by continuously monitoring fluorescence emission at 386 nm with excitation at 365 nm using a fluorimeter (LS50-B; Perkin-Elmer Corp., Norwalk, CT) after addition of 2 mM $MgCl_2$ and 100 mM KCl to initiate polymerization. The extent of polymerization was determined from the steady state fluorescence measured 12 h after polymerization was started.

Electron Microscopy

The polymerization conditions for negative staining at the electron microscopic level were identical to those described above. Actin (final concentration 3.3 μM) was allowed to polymerize with and without anti-αSM-1 IgG or Fab for 30 min. Samples were applied to carbon coated grids and stained with 1% uranyl acetate (Chaponnier et al., 1985).

Microinjection and Fluorescence Microscopy

SM cells were isolated from the thoracic aorta of 6-wk-old Wistar rats by enzymatic digestion (Skalli et al., 1986a) and used at passage 2–5. 10,000 cells were plated on 60-mm diam Petri dishes (Nunc; Gibco, Basel, Switzerland) and grown for 5–6 d in DME (Gibco), supplemented with 10% fetal calf serum. Microinjections were performed at room temperature by means of a Leitz micromanipulator (Leitz, Wetzlar, Germany), equipped with a vacuum and pressure device (Injectmatic, Geneva, Switzerland), using an inverted microscope equipped with phase contrast optics (Carl Zeiss, Inc., Oberkochen, Germany) as described earlier (Skalli et al., 1990). Cells were microinjected with the unlabeled or biotinylated α-SM actin NH_2-terminal decapeptide (AcEEEDSTALVC), or with the epitopic tetrapeptide (AcEEED) at 1 mg/ml in PBS; PBS alone or an α-SM actin related terminal peptide inactive in our tests (generally P2 [Table I]), were used as controls. After returning to the incubator for up to 5 h, cells were washed twice with PBS containing 0.5 mM $CaCl_2$ and 3 mM $MgCl_2$, fixed with 3% paraformaldehyde for 10 min and permeabilized with 0.1% Triton X-100 in PBS for 1 min. Double immunofluorescence stainings were performed for α-SM actin and total actin using anti-αSM-1 (Skalli et al., 1986b) and a rabbit polyclonal general anti-actin antibody, respectively (Skalli et al., 1990), followed by TRITC- or FITC-conjugated goat anti–mouse IgG (Jackson) and FITC-conjugated goat anti–rabbit IgG (Jackson). Rhodamine-labeled phalloidin (Molecular Probes, Inc., Eugene, OR) was also used to detect total polymerized actin instead of anti-actin general antibody. The biotinylated peptide was detected by FITC-streptavidin (F 422; Dako A/S, Glostrup, Denmark). After several rinsings in

Table I. Blocking Effect of NH₂-terminal Actin Peptides and Related Synthetic Peptides on Anti–αSM-1 Binding*

P1	Ac. EEEDSTALVC.OH	α-smooth	Yes
P2	Ac. DEDETTALVC.OH	α-skeletal	No
P3	Ac. DDEETTALVC.OH	α-cardiac	No
P4	Ac. EEETTALVC.OH	γ-smooth	No
P5	Ac. EEEIAALVC.OH	γ-cytoplasmic	No
P6	Ac. DDDIAALVC.OH	β-cytoplasmic	No
P7	NH₂.EEEDSTALVC.OH		No
P8	But.EEEDSTALVC.OH		No
P9	Ac. _EEDSTALVC.OH		No
P10	Ac. DEEDSTALVC.OH		No
P11	Ac. AEEDSTALVC.OH		Partial
P12	Ac. EDEDSTALVC.OH		No
P13	Ac. EAEDSTALVC.OH		No
P14	Ac. EEDDSTALVC.OH		No
P15	Ac. EEADSTALVC.OH		No
P16	Ac. EEEESTALVC.OH		Partial
P17	Ac. EEEGSTALVC.OH		No
P18	Ac. EEEDATALVC.OH		Yes
P19	Ac. EEEDLTALVC.OH		Yes

*The amino acids in bold characters are those differing from the sequence of the α-SM actin NH₂-terminal peptide (P1).

PBS, the preparations were mounted in buffered polyvinyl alcohol (Lennett, 1978). Photographs were taken with a Zeiss Axiophot photomicroscope (Carl Zeiss) equipped for epiillumination and specific filters for fluorescein and rhodamine, using a plan apochromatic 63×/1.4 objective and Kodak T-MAX 400 film (Eastman Kodak, Rochester, NY).

Results

The Ac-EEED Sequence Constitutes the Epitope Recognized by Anti–α-SM-1

The monoclonal antibody anti-αSM-1 was originally obtained after sensitization with the NH₂-terminal decapeptide of α-SM actin (Skalli et al., 1986b). The corresponding free peptide (Table I, peptide P1) specifically blocked specific α-SM actin detection during immunofluorescence staining of different mammalian tissues. A similar inhibitory effect was also observed when a rat aorta protein total extract was screened by immunoblotting in the presence of the decapeptide (Fig. 1). Peptides corresponding to the NH₂-terminal sequences of other vertebrate actins (Table I, peptides P2–P6) were inactive when used as inhibitors in both immunofluorescence and Western blotting (Fig. 1).

To obtain more details on the amino acid residues involved in the interaction with the antibody, we first synthesized a number of variants of P1, in which either the
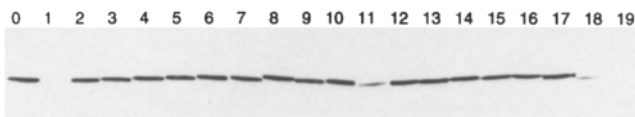

Figure 1. Blocking effect of NH₂-terminal peptides on anti–αSM-1. Rat media total extracts (3 μg/lane) were run on 5–15% SDS-PAGE and transferred on nitrocellulose membrane. Nitrocellulose strips were incubated with anti–αSM-1 hybridoma supernatant (1:500) alone (control, lane 0) or mixed with the peptides (10 μg/ml) listed in Table I (lane P1–P19). Only the α-SM NH₂-terminal peptide (P1) and peptides with substitution in position 5 (P18 and P19) fully blocked the immunoreactivity of anti–αSM-1.

acetyl group was deleted or substituted by a more bulky butyryl group (Table I, P7 and P8). Both peptides were unable to compete with α-SM actin for the antibody, stressing the importance of the presence of the acetyl group at the NH₂-terminal end. The remaining peptides were designed by substituting at positions 1–4 the acidic residues by either the other acidic side chain (exchanging Asp for Glu and vice-versa) or a small neutral residue (either Ala or Gly). At position 5 the serine residue was replaced by either a small (Ala) or bulky (Leu) hydrophobic residue (Table I, P10-P19). The binding of anti-αSM-1 to α-SM actin was never blocked when substitutions, including conserved substitutions (i.e., Glu by Asp), happened in position 2 or 3. Interestingly, exchanging Glu by the uncharged Ala in position 1 resulted in a partial blocking of anti–αSM-1 binding while substituting Glu by Asp had no effect. In position 4, however, only the conserved substitution Asp for Glu resulted in a partial effect. Finally, peptides with modifications at position 5 (P18 and P19) blocked the antibody reaction with the same efficiency as the natural peptide. In addition, full inhibition was seen with the acetylated and amidated tetrapeptide Ac-EEED-NH₂ (not shown). These data are consistent with the possibility that the epitope recognized by anti–αSM-1 is restricted to the first four amino acids of α-SM actin and includes the NH₂-terminal acetyl moiety.

The same specific interactions between the antibody and the peptides were also measured in an experiment in which the peptides, now conjugated to bovine serum albumin as carrier, were used as targets. The different conjugates were run on SDS-PAGE gels and transferred onto nitrocellulose membranes. Then they were probed with anti–αSM-1 as primary antibody in a classical Western blotting technique (Fig. 2). All peptides that were able to either completely or partially block α-SM actin antibody interaction in their free form were now specifically recognized as BSA-conjugates in Western blotting (Fig. 2). Those that did not display any inhibitory activity as free peptides were also not detected by immunoblotting.

Effect of Anti-αSM-1 on α-SM Actin Polymerization In Vitro

We further investigated whether the epitope recognized by anti–αSM-1 is involved in α-SM actin polymerization, as it is known that a number of actin regulatory proteins can be chemically cross-linked via the NH₂ terminus of actin (Vandekerckhove, 1990). This was done by measuring the extent of actin polymerization by a sedimentation assay and subsequent quantification of the protein present in the pellets and supernatants using SDS-PAGE (Chapon-
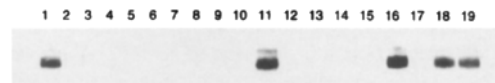

Figure 2. Detection of sequences recognized by anti–αSM-1 in denatured NH₂-terminal peptides. Peptides listed in Table I were coupled to BSA, run on SDS-PAGE and transferred on nitrocellulose membrane. Anti–αSM-1 reacted with the α-SM NH₂-terminal peptide (P1), a peptide modified in position 1 (Ala for Glu) (P11), a peptide modified in position 4 (Glu for Asp) and with the two peptides having substitution in position 5 (P18 and P19).

nier et al., 1985) and by the pyrene-actin fluorescence assay of Kouyama and Mihashi (1981).

Aortic SM actin at 3.3 μM final concentration was, as expected, exclusively recovered in the supernatant of low salt buffer (buffer G) (Fig. 3 A, condition 1). In physiological salt conditions (100 mM KCl, 2 mM MgCl₂ final concentration), only 10–20% of SM actin was recovered in the pellet fraction (Fig. 3 A, condition 2). However, when actin was mixed with anti–αSM-1 (1.6 μM final concentration) in the same salt conditions, polymerization was induced, with more than 80% of the protein recovered in the pellet fraction together with the antibody (Fig. 3 A, condition 3). The antibody did not induce α-SM actin polymerization in low salt buffer (Fig. 3 A, condition 4). As a control it was shown that α-SK actin polymerized normally in physiological conditions and that there was no additional polymerization when anti–αSM-1 was mixed with α-SK actin in a 1:2 antibody/actin molar ratio (Fig. 3 B). The antibody was effective even substoichiometrically, as more than 60% of actin was recovered in the pellet fraction at a 1:16 molar ratio (data not shown). Control experiments showed that an unrelated IgG had no effect on actin polymerization (data not shown).

We further measured the effect of the antibody on α-SM actin lower than 3.3 μM, using a 1:2 antibody/actin molar ratio. At a 0.8 μM actin concentration, the antibody was still effective in inducing polymerization, as 50% of actin was recovered in the pellet fraction, although in these conditions actin alone was hardly polymerizable (data not shown). The polymerization effect of anti–αSM-1 through its binding to AcEEED sequence was further proved by performing experiments in the presence of excess peptides (Fig. 4). Only the α-SM decapeptide (P1) and the epitopic tetrapeptide AcEEED inhibited the antibody effect on actin assembly, leaving the antibody in the supernatant frac-
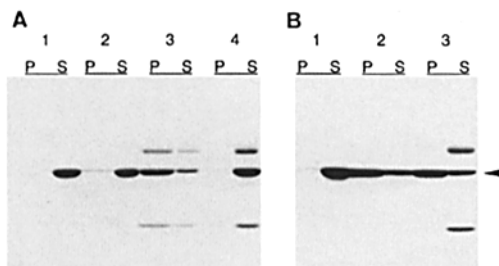

Figure 4. AcEEED containing peptides inhibit the polymerization effect of anti–αSM-1. α-SM decapeptide P1 (10 μg condition 2; 2.5 μg condition 3), α-SK decapeptide P2 (condition 4), AcEEED (condition 5) or EEED (condition 6) were incubated with 12.5 μg of anti–αSM-1 for 10 min before adding actin and salt for the 30 min polymerization time as described in materials and methods section and in Fig. 3. The supernatants and pellets after airfuge centrifugation were analyzed on Coomassie blue SDS-PAGE gel. Anti-αSM-1 with the bulk of actin were recovered in the pellet fraction in the control condition (no peptide added, condition 1) and when α-SK decapeptide P2 (condition 4) or the unacetylated tetrapeptide EEED were preincubated with the antibody. The α-SM decapeptide P1 (condition 2 and 3) as well as the acetylated tetrapeptide AcEEED (condition 5) inhibited the polymerization effect of anti–αSM-1, keeping the antibody in the supernatant fraction.

tion (conditions 2, 3, and 5). The α-SK actin decapeptide (P2) or the unacetylated tetrapeptide EEED did not inhibit the polymerizing effect of the antibody (conditions 4 and 6), resulting in a recovery of anti–αSM-1 in the pellet together with the bulk of polymerized actin as in the positive control (condition 1, antibody alone).

Anti–αSM-1 Fab, containing a single actin-binding site, showed the same effect as the complete antibody molecule, excluding the possibility that α-SM actin pelleting was due to aggregation by anti–αSM-1. This point was controlled at the electron microscopic level using negative staining of anti–αSM-1–induced actin filaments. As shown in Fig. 5, typical F-actin filaments were seen and no aggregated actin polymers were apparent.

Figure 3. Sedimentation assays. Effect of anti–αSM-1 on α-SM actin polymerization. (A) SM actin (3.3 μM final) alone (1 and 2) or mixed with anti–αSM-1 (1.6 μM final) (3 and 4) without (1 and 4) or with (2 and 3) salts (100 mM KCl and 2 mM MgCl₂) was incubated for 30 min at room temperature and centrifuged at 30 PSI in a Beckman airfuge for 1 hour. Coomassie blue-stained gels of pellet (P) and supernatant (S) showed that SM actin is totally recovered in the supernatant when alone in buffer A without salt (1). When salt was added, only a small proportion of actin polymerized (2). The proportion of actin recovered in the pellet strongly increased when anti–αSM-1 was added to actin in polymerizing conditions (3). The antibody was distributed proportionally to actin in the two fractions (P and S). In depolymerizing conditions, anti–αSM-1 had no influence on actin polymerization (4). (B) Skeletal actin (3.3 μM final) did not polymerize in the absence of salts (1) and polymerized in the presence of salts (2). Anti-αSM-1 added to α-skeletal actin in polymerizing conditions had no effect on polymerization and was recovered in the supernatant fraction (3).
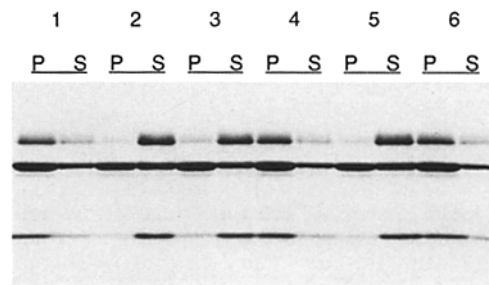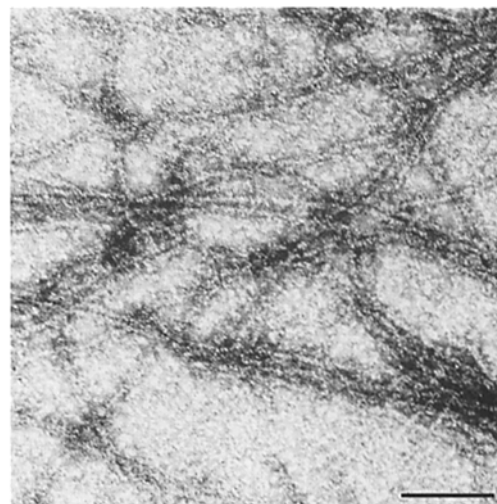

Figure 5. Electron micrograph of negatively stained typical actin filaments induced by anti–αSM-1 in polymerizing buffer (same condition as in Fig. 3). Bar, 0.1 μm.

Pyrene-Actin Experiments

Fig. 6 A shows the rate of fluorescence increase after adding salts to a solution containing 200 nM pyrene-labeled α-SM actin and 3.3 μM unlabeled α-SK or α-SM actin in the presence or absence of a 1:2 ratio of anti–αSM-1. In the absence of the antibody, the fluorescence of the trace labeled α-SM actin did not increase during the 20 min of measurement. The slight decrease was due to photobleaching of the probe, which likely occurs because of the high illumination required to detect the small concentration of labeled actin. In contrast, in the presence of the antibody, the fluorescence increased at a rate that was greater than the rate seen in the presence of 3.3 μM α-SK actin. The slowness of the fluorescence change at very high anti–αSM-1 concentration is inconsistent with the antibody acting as an efficient nucleation factor (Fig. 6 A). Fig. 6 B shows that the α-SM actin antibody affected the steady-state fluorescence levels as well as the rate of fluorescence change. The fluorescence intensity of α-SM actin in the presence of saturating amounts of anti–αSM-1 were nearly identical to those of α-SK actin, indicating that under these conditions, the two actin isoforms have similar critical monomer concentrations (estimated to be 200 nM). In
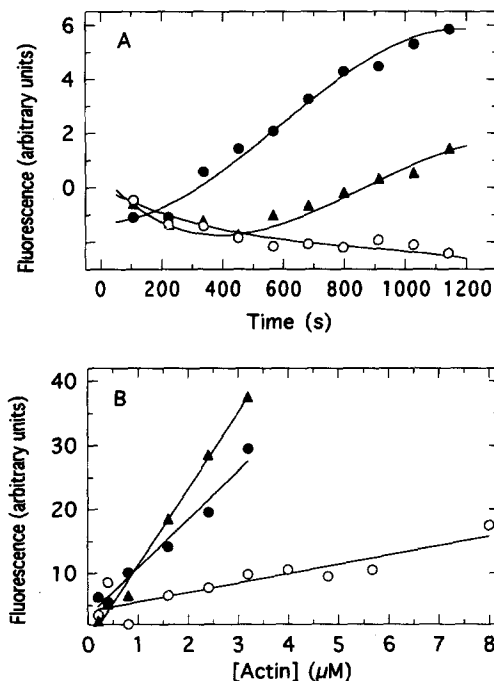
the absence of antibody, the fluorescence intensity of α-SM actin was much lower, consistent with a critical concentration of several μM. The effect of the antibody on α-SM actin fluorescence is due to polymerization of the actin rather than to a direct effect of the antibody on the fluorescence of the labeled actin monomers, because the antibody had a similar effect on α-SM actin mixed with a trace of pyrene-labeled α-SK actin (data not shown). Since the antibody does not bind to α-SK actin, the fluorescence intensity of this actin can increase only if it incorporates into a complex with the α-SM actin that has the fluorescence properties of an actin filament.

In conclusion, the binding of anti–αSM-1 to the AcEEED sequence of aortic actin likely induces a conformational change that converts monomeric actin into a polymerization competent form. The binding does not hinder the incorporation of the actin-antibody complex in the filaments.

Action of Ac-EEED Microinjection on Stress Fiber Composition in Cultured SM Cells

Our findings, that the critical monomer concentration of α-SM actin is decreased with the NH_2 terminus-specific antibody, raise the possibility that an excess of the AcEEED peptide within the cell interferes with α-SM actin polymerization and incorporation into stress fibers. To test this possibility, both peptide P1 and epitopic tetrapeptide (AcEEED-NH_2), were microinjected into cultured SM cells and the actin organization was subsequently analyzed by double immunostaining using a total actin-specific polyclonal rabbit antibody and anti–αSM-1.

Microinjection of PBS or nonblocking peptides diluted at 1 mg/ml into SM cells had no effect on actin organization; double staining for total actin and α-SM actin revealed the classical actin stress fiber organization in untreated as well as in buffer injected SM cells (Fig. 7, a and b). On the other hand, a striking change was observed within 30 min to 5 h after microinjection of the peptide. Although the antibody recognizing all actin isoforms still decorated stress fibers in a normal pattern (Fig. 7 c), anti–αSM-1 failed to stain actin filaments or stained them only faintly (Fig. 7 d). In the same microscopic field, neighboring noninjected cells showed typical stress fibers decorated by both antibodies (Fig. 7, c and d). Interestingly, the earliest changes of α-SM actin organization were only observed about 30 min after injection. When fixed and permeabilized cells were incubated with the active peptides before double staining, no effect on α-SM actin organization was noted. These results suggest that in vivo, a dynamic turnover of actin in stress fibers is necessary for the peptides to be active.

Cells that were fixed 1–6 min after microinjection did not show an altered stress fiber organization revealed by either total actin or α-SM actin-specific antibody (Fig. 8, a and b). Within 1–6 min after microinjection, the biotinylated decapeptide appeared localized in α-SM actin containing stress fibers (Fig. 8, c and d) and disappeared later. When the biotinylated NH_2-terminal peptide of α-skeletal actin was microinjected, it did not localize in stress fibers. These observations indicate that the failure of α-SM actin staining 30 min to 5 h after peptide injection is not simply

Figure 6. Pyrene actin assays. (*A*) Effect of anti–αSM-1 on kinetics of α-SM actin polymerization. 200 nM pyrene-labeled α-SM actin was mixed with 3.1 μM unlabeled α-SK actin in G-actin buffer (*triangles*) or with 3.1 μM unlabeled α-SM actin with (*closed circles*) or without (*open circles*) anti–αSM-1 at a 1:2 antibody/actin ratio. The salt concentration was raised to 2 mM $MgCl_2$ and 100 mM KCl by addition of a 10× concentrated stock, and the polymerization is shown as an increase in pyrene-fluorescence. (*B*) Effect of anti–αSM-1 on the final extent of actin polymerization. The fluorescence, proportional to the amount of polymerized actin, of samples such as those shown in Fig. 6 *A* but with various concentrations of unlabeled actins was measured 12 h after initiation of polymerization. Symbols have the same meaning as in Fig. 6 *A*.
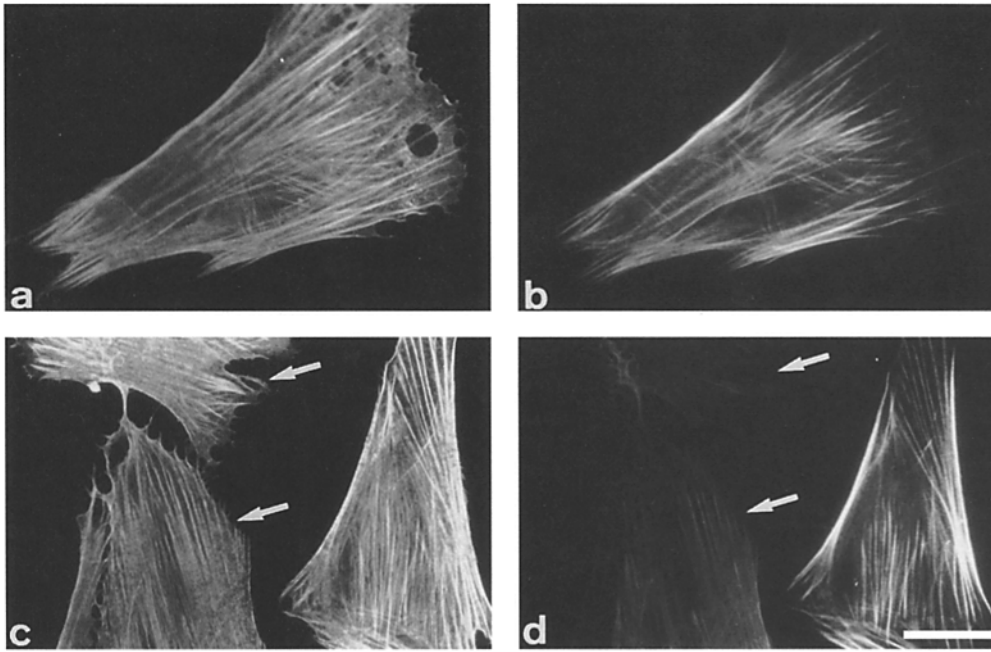
Figure 7. Double immuno-fluorescence staining for total actin (*a* and *c*) and for α-SM actin (*b* and *d*) of cultured rat aortic SM. 2 h after PBS microinjection, the pattern of stress fiber stainings for total actin (*a*) and for α-SM actin (*b*) are similar to those of noninjected cells. Although total actin (*a*) and α-SM actin (*b*) are mainly colocalized, a more extended staining for total actin is observed, with longer stress fibers and diffuse cytoplasmic staining. In cells microinjected with the NH₂-terminal peptide P1 (*arrows*) the pattern of stress fiber staining for total actin is similar to that of noninjected neighboring cells (*c*) but, stress fiber staining for α-SM actin is practically abolished (*d*). Bar, 10 μM.

due to saturation of the antibody by the peptide still present after permeabilization and fixation procedures. Our results after microinjection of the biotinylated AcEEED peptide (Fig. 8, *c* and *d*), indicate that this peptide does not make the NH₂ terminus of α-SM actin inaccessible for the antibody (Fig. 8 *d*). It is also noteworthy that peptide microinjection did not lead to complete stress fiber disruption, as shown by the unaltered total actin staining (Fig. 7 *c*), but that only the α-SM actin could not be detected anymore (Fig. 7 *d*).

Discussion

Anti-αSM-1 was the first monoclonal antibody described

to be specific for a single actin isoform (Skalli et al., 1986*b*); it has proved a reliable tool for the study of vascular SM cell and myofibroblast phenotypic modulations in physiological and pathological processes (Clowes et al., 1988; Desmoulière and Gabbiani, 1994; Grinnell, 1994). Our results suggest that: (*a*) AcEEED is the epitope recognized by anti–αSM-1, (*b*) in vitro, the antibody and its Fab fragment increase α-SM actin polymerization (conceivably by lowering the critical monomer concentration), (*c*) in cultured SM cells, the epitopic sequence plays a role in α-SM actin polymerization and stress fiber organization.

Incubation of synthetic peptides with anti–αSM-1 showed that aspartic acid (D) in position 4 is essential for antibody recognition, as it represents the only difference between
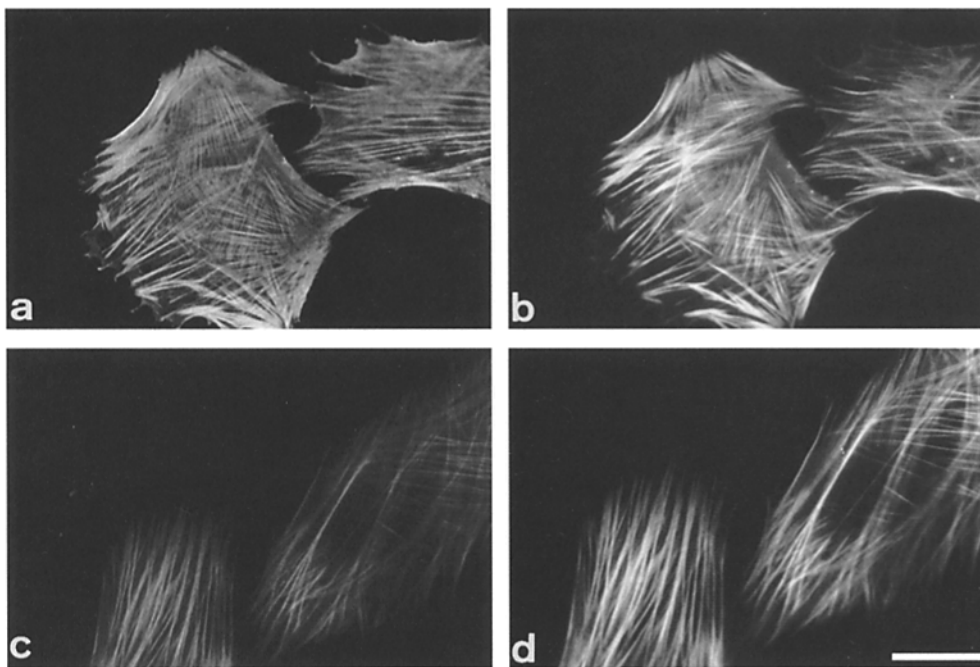

Figure 8. Double immuno-fluorescence staining for total actin (*a*) and for α-SM actin (*b*), for the biotinylated α-SM actin NH₂-terminal peptide (*c*) and for α-SM actin (*d*) 1–6 min after peptide microinjection. The patterns of stress fiber staining for total actin (*a*) and for α-SM actin (*b*) in P1 microinjected SM cells are similar to those of non-injected neighboring cells. The biotinylated NH₂-terminal decapeptide as revealed by FITC-streptavidin (*c*) is detected along stress fibers, colocalized with α-SM actin staining (*d*). Bar, 10 μM.

the NH$_2$-terminal end of α-SM actin and those of γ-SM (AcEEET) or γ-cytoplasmic (AcEEEI) actins. The acetyl blocking group also appears to be an integral part of the epitope. A search in the Swissprot data bank indicated that more than 300 mammalian proteins contain an EEED sequence not located at the NH$_2$ terminus. Thus the specificity of our antibody not only relies on the order and nature of the four amino acids but also on their relative position with respect to the blocking acetyl group.

The sequence divergences among NH$_2$-terminal ends of actin isoforms make this portion a very likely candidate for specific interactions. Indeed a large number of actin-binding proteins (ABP) have been proposed to interact with the NH$_2$-terminal domain (Vandekerckhove, 1990); as far as we know, none of them has been shown to specifically interact with a single isoform. Most of these studies were based on cross-linking or mutation techniques and surprisingly utilized mainly α-skeletal actin as test partner, not taking into account the well known NH$_2$-terminal sequence divergences among actin isoforms. Many ABP or their fragments containing the actin-binding site have been shown in vitro to have an actin polymerizing effect (Lewis et al., 1983; Vandekerckhove, 1990; Eto et al., 1991; Hartwig and Kwiatkowski, 1991; Wang et al., 1991; Friederich et al., 1992; Mannherz, 1992; Vancompernolle et al., 1992; Vandekerckhove and Vancompernolle, 1992; Reisler, 1993). Most interactions between actin and ABP apparently involve salt bridges between the acidic residues of actin and basic domains of ABP (Vandekerckhove and Vancompernolle, 1992). However, it has been suggested that these interactions with NH$_2$-terminal residues are charge and not sequence dependent (Highsmith, 1990; Chase et al., 1991). The importance of ATP hydrolysis in the control of the actin monomer-polymer steady-state has been suggested in experiments where profilin was shown to promote actin filament assembly from the pool of actin-thymosin β4 complex (Pantaloni and Carlier, 1993). When compared to the above mentioned reports, our findings deserve some comments: (a) anti–αSM-1 binding is sequence specific, and has a polymerization effect only on α-SM actin; (b) the enhanced α-SM actin polymerization by anti–αSM-1 takes place only in physiological salt conditions. On the contrary, actin polymerization by the above mentioned ABP and/or actin-binding domains was promoted essentially in low salt condition (Eto et al., 1991; Friederich et al., 1992; Vancompernolle et al., 1992); addition of salt even reduced this effect, thus reinforcing the possibility of an electrostatic force interaction.

We have no straightforward explanation about the actin polymerizing activity of anti–αSM-1. No three-dimensional model of α-SM actin is presently available, but there is no reason to suspect that it is very different from the available α-skeletal (Kabsch et al., 1990) or β-cytoplasmic actin models (Schutt et al., 1993). The NH$_2$-terminal end in polymerized α-SM actin is probably exposed as indicated by the binding of anti–αSM-1 by immunofluorescence, similarly to what has been suggested for α-skeletal actin (Orlova et al., 1994). Our results suggest that anti–αSM-1 binding to α-SM actin may result in a conformational change making it more competent for polymerization. If the COOH-terminal is in close vicinity to the NH$_2$-terminal end of the molecule (Kabsch et al., 1990), the antibody

binding could disrupt some electrostatic bonds and render α-SM actin more polymerizable by slightly changing the molecular conformation. Possibly our data cannot be interpreted solely in terms of critical concentration. We have recently obtained evidence from both fluorescence microscopy of rhodamine-phalloidin–labeled F-actin and from dynamic light scattering that α-SM actin even at high concentrations forms short filaments compared to those formed by α-SK actin under the same conditions (unpublished observations). It is conceivable that anti–αSM-1 increases the average filament length (as we observed by light scattering) and this effect alone or in addition to changes in the G/F actin ratio, contributes to the rise in fluorescence.

We have further explored the role of the α-SM actin NH$_2$-terminal peptide by microinjecting it in living cells. Disruption of stress fibers in nonmuscle cells has been observed after microinjection of several ABP (Füchtbauer et al., 1983; Cooper et al., 1987; Sanger et al., 1987, 1990; Franck et al., 1990; Huckriede et al., 1990; Pavalko and Burridge, 1991; Cao et al., 1992; Sanders et al., 1992; Ranucci et al., 1993). We have reported earlier that injection of two actin antibodies, both directed against the NH$_2$-terminal end of actin but with different specificities (anti–αSM-1 and a rabbit polyclonal antibody recognizing SM and cytoplasmic actin isoforms) affect in a different manner stress fiber organization in SM cells (Skalli et al., 1990). Microinjection of the general antibody abolished most of the stress fiber staining by rhodamine-phalloidin, while injection of anti–αSM-1 left practically intact the rhodamine-phalloidin staining, but displaced α-SM actin staining from stress fibers to a perinuclear, spotty distribution (Skalli et al., 1990). This result suggests that microinjected anti–αSM-1 interferes with the α-SM actin organization in stress fibers, yet retaining it within the cytoplasm in the form of small aggregates. Our present results showed a selective localization of the peptide on stress fibers shortly after microinjection and a practically complete disappearance of anti–αSM-1 staining starting at $\sim$30 min after the injection of the α-SM actin NH$_2$-terminal tetra- or decapeptides. Several mechanisms may account for this effect, e.g., interference with actin acetylation or modification of stress fiber organization with loss of the epitope availability at the surface of the filaments. An additional attractive possibility is that the peptide binds to a yet unknown protein that contains a sequence similar to that present in the antibody and helps the incorporation of α-SM actin into stress fibers during the process of filament turnover in these cells. Further work is necessary to define the exact molecular mechanism of peptide stress fiber interactions.

Cytoskeletal proteins are highly compartmentalized; this is probably true also for actin isoforms (Herman, 1993; Gimona et al., 1994; North et al., 1994). Although most work so far favors a coexpression of actin isoforms in single microfilaments (Otey et al., 1986), no absolute evidence of this possibility is presently available. Actin has been shown to exchange dynamically in cells like fibroblasts from the G- to the F-pool and vice versa (Wang, 1984). Various experimental approaches (Kreis et al., 1982; Amato and Taylor, 1986; Pavalko and Burridge, 1991; Theriot and Mitchison, 1991) have shown that the

rate of polymer turnover is very rapid. The average half-life of actin in stress fibers was measured between 3.8 min using photoactivation of caged resorufin actin (Theriot and Mitchison, 1991) and 10 min using fluorescence microscopy after photobleaching (Kreis et al., 1982). Thinner stress fibers turn over more rapidly than thicker ones (Theriot and Mitchison, 1991). Taking into account the rate of stress fiber turnover and the fact that any actin isoform can participate in stress fiber formation, it is conceivable that filaments detected by the total actin antibody after peptide injection are newly formed by actin isoforms other than α-SM actin.

In conclusion our work has shown an hitherto unknown function of the NH$_2$-terminal sequence AcEEED of α-SM actin. Moreover, our microinjection experiments suggest that the peptide AcEEED may be utilized for the study of the function of this α-SM actin in cells such as SM cells, fibroblasts, myofibroblasts, and myoepithelial cells in physiological and pathological situations during which α-SM actin expression is modulated. The function of this actin isoform has interested our and other laboratories for many years (Desmoulière and Gabbiani, 1994; Grinnell, 1994) and up to now has been approached only indirectly.

References

Amato, P. A., and D. L. Taylor. 1986. Probing the mechanism of incorporation of fluorescently labeled actin into stress fibers. *J. Cell Biol.* 102:1074–1084.

Cao, L.-G., G. G. Babcock, P. A. Rubenstein, and Y.-L. Wang. 1992. Effects of profilin and profilactin on actin structure and function in living cells. *J. Cell Biol.* 117:1023–1029.

Chaponnier, C., P. Patebex, and G. Gabbiani. 1985. Human plasma actin-depolymerizing factor: purification, biological activity and localization in leukocytes and platelets. *Eur. J. Biochem.* 146:267–276.

Chaponnier, C., O. Kocher, and G. Gabbiani. 1990. Modulation of gelsolin content in rat aortic smooth muscle cells during development, experimental intimal thickening and culture. An immunohistochemical and biochemical study. *Eur. J. Biochem.* 190:559–565.

Chase, P. B., T. W. Beck, J. Bursell, and M. J. Kushmerick. 1991. Molecular charge dominates the inhibition of actomyosin in skinned muscle fibers by SH₁ peptides. *Biophys. J.* 60:352–359.

Clowes, A. W., M. M. Clowes, O. Kocher, P. Ropraz, C. Chaponnier, and G. Gabbiani. 1988. Arterial smooth muscle cells in vivo: relationship between actin isoform expression and mitogenesis and their modulation by heparin. *J. Cell Biol.* 107:1939–1945.

Cooper, J. A., J. Bryan, B. I. Schwab, C. Frieden, D. J. Loftus, and E. L. Elson. 1987. Microinjection of gelsolin into living cells. *J. Cell Biol.* 104:491–501.

Desmoulière, A., and G. Gabbiani. 1994. Modulation of fibroblastic cytoskeletal features during pathological situations: the role of extracellular matrix and cytokines. *Cell Motil. Cytoskeleton.* 29:195–203.

Eto, M., F. Morita, N. Nishi, S. Tokura, T. Ito, and K. Takahashi. 1991. Actin polymerization promoted by a heptapeptide, an analog of the actin-binding S site on myosin head. *J. Biol. Chem.* 266:18233–18236.

Franck, Z., M. Footer, and A. Bretscher. 1990. Microinjection of villin into cultured cells induces rapid and long-lasting changes in cell morphology but does not inhibit cytokenesis, cell motility or membrane ruffling. *J. Cell Biol.* 111:2475–2485.

Friederich, E., K. Vancompernolle, C. Huet, M. Goethals, F. Finidori, J. Vandekerckhove, and D. Louvard. 1992. An actin-binding site containing a conserved motif of charged amino acid residues is essential for the morpho-

genic effect of villin. *Cell.* 70:81–92.

Füchtbauer, A., B. M. Jockusch, H. Maruta, M. W. Kilimann, and G. Isenberg. 1983. Disruption of microfilament organization after injection of F-actin capping proteins into living tissue cells. *Nature (Lond.).* 304:361–364.

Gimona, M., J. Vandekerckhove, M. Goethals, M. Herzog, Z. Lando, and J. V. Small. 1994. β-actin specific monoclonal antibody. *Cell Motil. Cytoskeleton.* 27:108–116.

Green, M., H. Alexander, A. Olson, S. Alexander, T. M. Shinnick, J. G. Sutcliffe, and R. A. Lerner. 1982. Immunogenic structure of the influenza virus hemagglutinin. *Cell.* 28:477–487.

Grinnell, F. 1994. Fibroblasts, myofibroblasts, and wound contraction. *J. Cell Biol.* 124:401–404.

Hartwig, J. H., and D. J. Kwiatkowski. 1991. Actin-binding proteins. *Curr. Opin. Cell Biol.* 3:87–97.

Herman, I. M. 1993. Actin isoforms. *Curr. Opin. Cell Biol.* 5:48–55.

Highsmith, S. 1990. Electrostatic contributions to the binding of myosin and myosin-MgADP to F-actin in solution. *Biochemistry.* 29:10690–10694.

Huckriede, A., A. Füchtbauer, H. Hinssen, C. Chaponnier, A. Weeds, and B. M. Jockusch. 1990. Differential effects of gelsolins on tissue culture cells. *Cell Motil. Cytoskeleton.* 16:229–238.

Janmey, P. A., and C. Chaponnier. 1995. Medical aspects of the actin cytoskeleton. *Curr. Opin. Cell Biol.* 7:111–117.

Kabsch, W., H. G. Mannherz, D. Suck, E. F. Pai, and K. C. Holmes. 1990. Atomic structure of the actin: DNase I complex. *Nature (Lond.).* 347:37–44.

Kouyama, T., and K. Mihashi. 1981. Fluorimetry study of N-(1-pyrenyl)-iodoacetamide-labeled F-actin. *Eur. J. Biochem.* 114:33–38.

Kreis, T. E., B. Geiger, and J. Schlessinger. 1982. Mobility of rhodamine actin within living chicken gizzard cells determined by fluorescence photobleaching recovery. *Cell.* 29:835–845.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680–685.

Lennett, D. A. 1978. An improved mounting medium for immunofluorescence microscopy. *Am. J. Clin. Pathol.* 69:647–648.

Lewis, W. G., G. P. Cote, A. S. Mak, and L. B. Smillie. 1983. Amino acid sequence of equine platelet tropomyosin. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 156:269–273.

Mannherz, H. G. 1992. Crystallization of actin with actin-binding proteins. *J. Biol. Chem.* 267:11661–11664.

McKenna, N. M., J. B. Meigs, and Y.-L. Wang. 1985. Identical distribution of fluorescently labeled brain and muscle actins in living cardiac fibroblasts and myocytes. *J. Cell Biol.* 100:292–296.

North, A. J., M. Gimona, Z. Lando, and J. V. Small. 1994. Actin isoform compartments in chicken gizzard smooth muscle cells. *J. Cell Sci.* 107:445–455.

Orlova, A., X. Yu, and E. H. Egelman. 1994. Three-dimensional reconstruction of a co-complex of F-actin with antibody Fab fragments to actin's NH₂ terminus. *Biophys. J.* 66:276–285.

Otey, C. A., M. H. Kalnoski, J. L. Lessard, and J. C. Bulinski. 1986. Immunolocalization of the gamma isoform of nonmuscle actin in cultured cells. *J. Cell Biol.* 102:1726–1737.

Pantaloni, D., and M.-F. Carlier. 1993. How profilin promotes actin assembly in the presence of thymosin β4. *Cell.* 75:1007–1014.

Parham, P. 1986. Preparation and purification of active fragments from mouse monoclonal antibodies. *In* Handbook of Experimental Immunology. D. M. Weir, editor. Blackwell Scientific Publications, Berlin. 14.1–14.23.

Pavalko, F. M., and K. Burridge. 1991. Disruption of the actin cytoskeleton after microinjection of proteolytic fragments of α-actinin. *J. Cell Biol.* 114:481–491.

Ponte, P., P. Gunning, H. Blau, and L. Kedes. 1983. Human actin genes are single copy for α-skeletal and α-cardiac actin but multicopy for β- and γ-cytoskeletal genes: 3' untranslated regions are isotype specific but are conserved in evolution. *Mol. Cell Biol.* 3:1783–1791.

Ranucci, D., Y. Yamakita, F. Matsumura, and S. E. Hitchcock-DeGregori. 1993. Incorporation of microinjected mutant and wildtype recombinant tropomyosins into stress fibers in fibroblasts. *Cell Motil. Cytoskeleton.* 24:119–128.

Reisler, E. 1993. Actin molecular structure and function. *Curr. Opin. Cell Biol.* 5:41–47.

Ruzicka, D. L., and R. J. Schwartz. 1988. Sequential activation of α-actin genes during avian cardiogenesis: vascular smooth muscle α-actin gene transcripts mark the onset of cardiomyocyte differentiation. *J. Cell. Biol.* 107:2575–2586.

Sanders, M. C., A. L. Goldstein, and Y.-L. Wang. 1992. Thymosin β4 (Fx peptide) is a potent regulator of actin polymerization in living cells. *Proc. Natl. Acad. Sci. USA.* 89:4678–4682.

Sanger, J. M., B. Mittal, A. Wegner, B. M. Jockusch, and J. W. Sanger. 1987. Differential response of stress fibers and myofibrils to gelsolin. *Eur. J. Cell Biol.* 43:421–428.

Sanger, J. M., G. Dabiri, M. A. Mittal, M. A. Kowalski, J. G. Haddad, and J. W. Sanger. 1990. Disruption of microfilament organization in living nonmuscle cells by microinjection of plasma vitamin D–binding protein or DNase I. *Proc. Natl. Acad. Sci. USA.* 87:5474–5478.

Schutt, C. E., J. C. Myslik, M. D. Rozycki, N. C. W. Goonesekere, and U. Lindberg. 1993. The structure of crystalline profilin–β-actin. *Nature (Lond.).* 365:810–816.

Skalli, O., W. S. Bloom, P. Ropraz, B. Azzarone, and G. Gabbiani. 1986a. Cy-

toskeletal remodeling of rat aortic smooth muscle cells in vitro: relationships to culture conditions and analogies to in vivo situations. *J. Submicrosc. Cytol.* 18:481–493.

Skalli, O., P. Ropraz, A. Trzeciak, G. Benzonana, D. Gillessen, and G. Gabbiani. 1986b. A monoclonal antibody against α-smooth muscle actin: a new probe for smooth muscle differentiation. *J. Cell Biol.* 103:2787–2796.

Skalli, O., J. Vandekerckhove, and G. Gabbiani. 1987. Actin-isoform pattern as a marker of normal or pathological smooth-muscle and fibroblastic tissues. *Differentiation.* 33:232–238.

Skalli, O., F. Gabbiani, and G. Gabbiani. 1990. Action of general and α-smooth muscle-specific actin antibody microinjection on stress fibers of cultured smooth muscle cells. *Exp. Cell Res.* 187:119–125.

Spudich, J. A., and S. Watt. 1971. The regulation of rabbit skeletal muscle contraction. I. Biochemical studies of the interaction of the tropomyosin-troponin complex with actin and the proteolytic fragments of myosin. *J. Biol. Chem.* 246:4866–4871.

Strzelecka-Golaszewska, H., S. Zmorzynski, and M. Mossakowska. 1985. Bovine aorta actin. Development of an improved purification procedure and comparison of polymerization properties with actins from other types of muscle. *Biochim. Biophys. Acta.* 828:13–21.

Theriot, J. A., and T. J. Mitchison. 1991. Actin microfilament dynamics in locomoting cells. *Nature (Lond.).* 352:126–131.

Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA.* 76:4350–4354.

Vancompernolle, K., M. Goethals, C. Huet, D. Louvard, and J. Vandekerckhove. 1992. G- to F-actin modulation by single amino acid substitution in the binding site of actobindin and thymosin β4. *EMBO (Eur. Mol. Biol. Organ.) J.* 11:4739–4746.

Vandekerckhove, J. 1990. Actin-binding proteins. *Curr. Opin. Cell Biol.* 2:41–50.

Vandekerckhove, J., and K. Vancompernolle. 1992. Structural relationships of actin-binding proteins. *Curr. Opin. Cell Biol.* 4:36–42.

Vandekerckhove, J., and K. Weber. 1978. At least six different actins are expressed in a higher mammal: an analysis based on the amino acid sequence of the amino-terminal tryptic peptide. *J. Mol. Biol.* 126:783–802.

Vandekerckhove, J., and K. Weber. 1979. The complete amino acid sequence of actins from bovine aorta, bovine heart, bovine fast skeletal muscle, and rabbit slow skeletal muscle. *Differentiation.* 14:123–133.

Vandekerckhove, J., and K. Weber. 1981. Actin typing on total cellular extracts. A highly sensitive protein-chemical procedure able to distinguish different actins. *Eur. J. Biochem.* 113:595–603.

Wang, Y.-L. 1984. Reorganization of actin filament bundles in living fibroblasts. *J. Cell Biol.* 99:1478–1485.

Wang, C.-L. A., L.-W. C. Wang, S. Xu, R. C. Lu, V. Saavedra-Analis, and J. Bryan. 1991. Localization of the calmodulin- and the actin-binding sites of caldesmon. *J. Biol. Chem.* 266:9166–9172.

Woodcock-Mitchell, J., J. J. Mitchell, R. B. Low, M. Kieny, P. Sengel, L. Rubbia, O. Skalli, B. Jackson, and G. Gabbiani. 1988. α-smooth muscle actin is transiently expressed in embryonic rat cardiac and skeletal muscles. *Differentiation.* 39:161–166.