

# The NH<sub>2</sub>-terminal peptide of $\alpha$ -smooth muscle actin inhibits force generation by the myofibroblast in vitro and in vivo

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**M**yofibroblasts are specialized fibroblasts responsible for granulation tissue contraction and the soft tissue retractions occurring during fibrocontractive diseases. The marker of fibroblast-myofibroblast modulation is the neo expression of  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA), the actin isoform typical of vascular smooth muscle cells that has been suggested to play an important role in myofibroblast force generation. Actin isoforms differ slightly in their NH<sub>2</sub>-terminal sequences; these conserved differences suggest different functions. When the NH<sub>2</sub>-terminal sequence of  $\alpha$ -SMA Ac-EEED is delivered to cultured myofibroblast in the form of a fusion peptide (FP) with a cell penetrating sequence, it inhibits their contractile activity; moreover,

upon topical administration in vivo it inhibits the contraction of rat wound granulation tissue. The NH<sub>2</sub>-terminal peptide of  $\alpha$ -skeletal actin has no effect on myofibroblasts, whereas the NH<sub>2</sub>-terminal peptide of  $\beta$ -cytoplasmic actin abolishes the immunofluorescence staining for this isoform without influencing  $\alpha$ -SMA distribution and cell contraction. The FPs represent a new tool to better understand the specific functions of actin isoforms. Our findings support the crucial role of  $\alpha$ -SMA in wound contraction. The  $\alpha$ -SMA-FP will be useful for the understanding of the mechanisms of connective tissue remodeling; moreover, it furnishes the basis for a cytoskeleton-dependent preventive and/or therapeutic strategy for fibrocontractive pathological situations.

## Introduction

Myofibroblasts (Gabbiani et al., 1971; Majno et al., 1971) are specialized fibroblasts considered to be responsible for granulation tissue contraction (Martin, 1997) and soft tissue retractions typical of fibrocontractive diseases (Grinnell, 1994; Serini and Gabbiani, 1999). Their main activities are synthesis and remodeling of the extracellular matrix and generation of tensile force (Serini and Gabbiani, 1999). Acquisition of contractile properties by the myofibroblast is reflected in the neo expression of  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA),\* the actin isoform typical of vascular smooth muscle (SM) cells (Skalli et al., 1986; Darby et al., 1990) and, albeit less frequently, of other SM cell-specific proteins (e.g., SM myosin heavy chains, desmin, and caldesmon) (Serini and Gabbiani, 1999). The mechanisms of force gen-

eration by the myofibroblast are unknown presently but are clearly different than those playing a role in classical striated and SM contraction (Grinnell, 2000; Parizi et al., 2000). It is accepted that they depend on the formation and contraction of stress fibers (Burrige, 1981; Tomasek et al., 1992; Katoh et al., 1998), organelles typical of cultured fibroblasts, and of myofibroblasts in vivo (Serini and Gabbiani, 1999). The expression of  $\alpha$ -SMA in stress fibers increases the contractile activity of fibroblastic cells in vitro (Arora and McCulloch, 1994; Hinz et al., 2001a) and correlates with wound contraction efficiency in vivo (Darby et al., 1990; Hinz et al., 2001b). Actin, a major protein in all eukaryotic cells, is expressed in mammalian species as six cell type-specific isoforms. These isoforms show very small but conserved sequence differences (Vandekerckhove and Weber, 1978); their specific functions are presently not established. Actin isoforms differ essentially in their NH<sub>2</sub> terminus (Vandekerckhove and Weber, 1978); thus, this domain represents a likely candidate for specialized functions mediated through specific binding. The NH<sub>2</sub>-terminal sequence Ac-EEED (the epitope of the monoclonal antibody directed against  $\alpha$ -SMA) (Skalli et al., 1986; Chaponnier et al., 1995) appears to be important for  $\alpha$ -SMA polymerization and incorporation in stress fibers (Chaponnier et al., 1995). When microinjected

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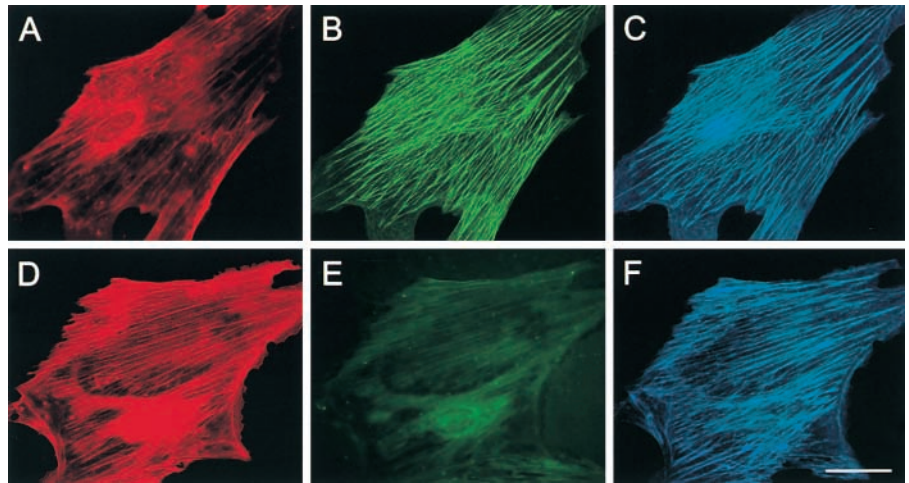
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\*Abbreviations used in this paper:  $\alpha$ -SMA,  $\alpha$ -smooth muscle actin;  $\beta$ -CA,  $\beta$ -cytoplasmic actin; ET, endothelin; FP, fusion peptide; LF, lung fibroblast; Rh, rhodamine; SKA, skeletal actin; SM, smooth muscle.

Key words: actin isoforms; wound healing; granulation tissue; TGF $\beta$ ; fibrosis

**Figure 1. Distribution of FPs in cultured LFs and their action on  $\alpha$ -SMA immunostaining.** SKA-Rh-FP (A, red) shows diffuse cytoplasmic distribution 30 min after application to the culture medium. At the same time, it does not affect the staining for  $\alpha$ -SMA (B, green) and  $\beta$ -CA (C, blue) in stress fibers. Under the same conditions, SMA-Rh-FP localizes along stress fibers (D) and abolishes  $\alpha$ -SMA staining (E) without changing the staining for  $\beta$ -CA (F). Bar, 50  $\mu$ m.



into cultured fibroblasts, Ac-EEED produces the disappearance of  $\alpha$ -SMA-specific immunodetection (Chaponnier et al., 1995), suggesting that it interferes with the function of this isoform. To verify whether the effects of peptide delivery include functional changes in myofibroblast activities, we have constructed a fusion peptide (FP; SMA-FP) including Ac-EEED and the Antennapedia third helix sequence (pAntp-Pro50) (Derossi et al., 1994) that allows cell penetration. We show here that the SMA-FP localizes in stress fibers rapidly after its delivery; it then inhibits contractility of myofibroblasts in vitro and granulation tissue contraction in vivo, thus supporting the assumption that  $\alpha$ -SMA plays an important role in wound contraction.

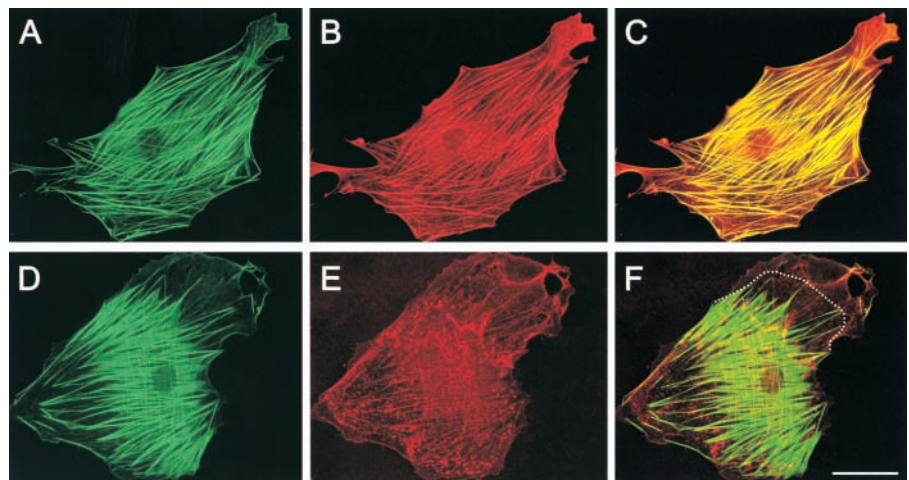
## Results

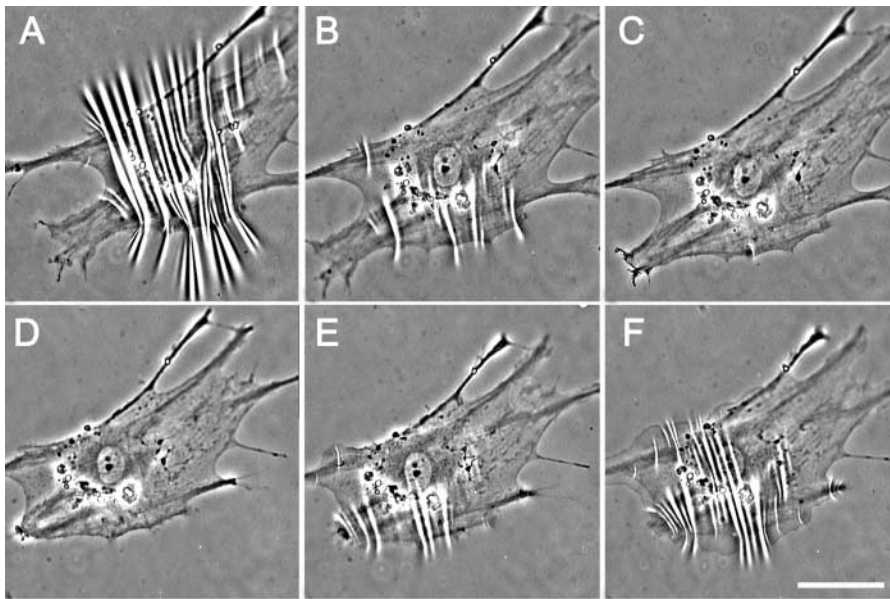
SMA-FP and the similarly constructed control  $\alpha$ -skeletal actin ( $\alpha$ -SKA) FP (SKA-FP), consisting of the NH<sub>2</sub>-terminal peptide of  $\alpha$ -SKA Ac-DEDE plus the vector pAntp-Pro50 (Derossi et al., 1994), were synthesized either without or with rhodamine (Rh) B labeling (SMA-Rh-FP and SKA-Rh-FP); when tested, labeling did not change FP activity (unpublished data). The  $\beta$ -cytoplasmic actin FP ( $\beta$ -CA-FP) was synthesized without Rh B labeling. We selected these controls because (a) the NH<sub>2</sub>-terminal se-

quence of  $\alpha$ -SKA is the closest to that of  $\alpha$ -SMA and (b)  $\beta$ -CA is highly expressed in myofibroblasts. When added to cultured rat lung fibroblasts (LFs) that express high levels of  $\alpha$ -SMA (Hinz et al., 2001a), the control SKA-Rh-FP showed within a few minutes a diffuse cytoplasmic distribution (Fig. 1 A) and had no effect on the immunostaining of  $\alpha$ -SMA (Fig. 1 B) and  $\beta$ -CA (Fig. 1 C). SMA-Rh-FP entered LFs similarly to SKA-Rh-FP but localized specifically in stress fibers (Fig. 1 D); the staining of stress fibers by the  $\alpha$ -SMA-specific antibody gradually decreased over 30 min (Fig. 1 E), whereas staining for  $\beta$ -CA remained unchanged (Fig. 1 F). After washing, the SMA-Rh-FP disappeared from stress fibers, and  $\alpha$ -SMA staining was restored over 30–60 min (unpublished data). These results suggest that Ac-EEED reversibly binds to a specific partner located in stress fibers. When the  $\beta$ -CA-FP was added to cultured LFs, it produced a gradual disappearance of  $\beta$ -CA from stress fibers over 30 min (Fig. 2 E) without any change in  $\alpha$ -SMA distribution (Fig. 2 D); moreover, large cytoplasmic protrusions devoid of filamentous  $\beta$ -CA and  $\alpha$ -SMA appeared regularly at the periphery of these cells (Fig. 2 F), suggesting that  $\beta$ -CA-FP modifies cellular shape by affecting the cortical network (Fig. 2).

To investigate the effect of SMA-FP on individual myofibroblasts, LFs were grown on deformable silicone substrates (Har-

**Figure 2. Effect of the  $\beta$ -CA-FP on cultured LFs.** Control LFs coexpress  $\alpha$ -SMA (A, green) and  $\beta$ -CA (B, red) in stress fibers (C, yellow). When added to cultured LFs for 30 min, the  $\beta$ -CA-FP does not affect the staining for  $\alpha$ -SMA (D) but abolishes staining for  $\beta$ -CA (E). Moreover,  $\beta$ -CA-FP induces the de novo formation of lamellar protrusions that are free of stress fibers (F, white line indicates putative cell edge before treatment). Bar, 50  $\mu$ m.





**Figure 3. SMA-FP inhibits the tension exerted by LFs on silicone substrates.**

(A) Untreated LFs produce wrinkles on deformable silicone substrates during 60 min recording. (B) Wrinkles decrease in number already 15 min after treatment with SMA-FP and completely disappear after 30 min (C). (D) 10 min after removal of the SMA-FP by repeated washing, LFs contract again followed by gradual wrinkle reformation after 30 (E) and 60 min (F). Bar, 50  $\mu$ m. Also see the video available at <http://www.jcb.org/cgi/content/full/jcb.200201049/DC1>.

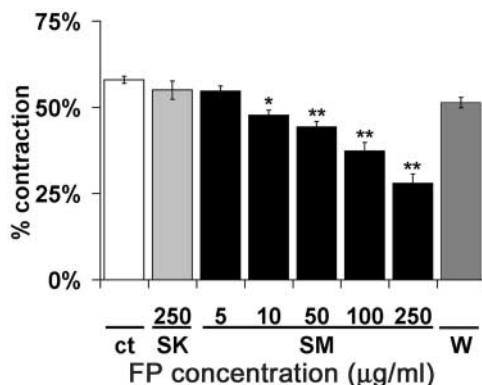
ris et al., 1980), which were produced with a stiffness that restricts the formation of wrinkles to highly contractile  $\alpha$ -SMA-positive fibroblasts (Hinz et al., 2001a) (Fig. 3 A; video available at <http://www.jcb.org/cgi/content/full/jcb.200201049/DC1>). At a concentration of 5–10  $\mu$ g/ml, SMA-FP caused a release of cellular tension as visualized by the reduction in the number of wrinkles. This action was visible within 15 min (Fig. 3 B) and maximal at 30 min (Fig. 3 C). Removing the SMA-FP reversed this effect: after 10 min of washing, LFs first retracted as indicated by a reduction of their area (Fig. 3 D). This was followed by wrinkle reformation after 30–60 min (Fig. 3, E and F). The cycle of wrinkle disappearance and reappearance was repeated up to three times. SKA-FP and  $\beta$ -CA-FP had no effect at all concentrations tested (unpublished data).

Attached collagen lattices allow the quantitative assessment of the contractile activity of cell populations when released

from the culture dish (Grinnell, 1994). LF-populated lattices contracted rapidly, and after 30 min their diameters were reduced maximally to 58% of the initial diameter (Fig. 4, ct). SKA-FP had no effect on lattice contraction (Fig. 4, SK, 250  $\mu$ g/ml). SMA-FP reduced lattice contraction concentration dependently (Fig. 4 SM, from 56% at 5  $\mu$ g/ml to 27% of the initial diameter at 250  $\mu$ g/ml). Washing out the SMA-FP before lattice release resulted in control contraction levels (Fig. 4, W). The specific inhibitory effect of SMA-FP on tension production by LFs suggests a role of  $\alpha$ -SMA-mediated myofibroblast contraction. Its reversibility indicates that  $\alpha$ -SMA-FP does not affect significantly cell integrity.

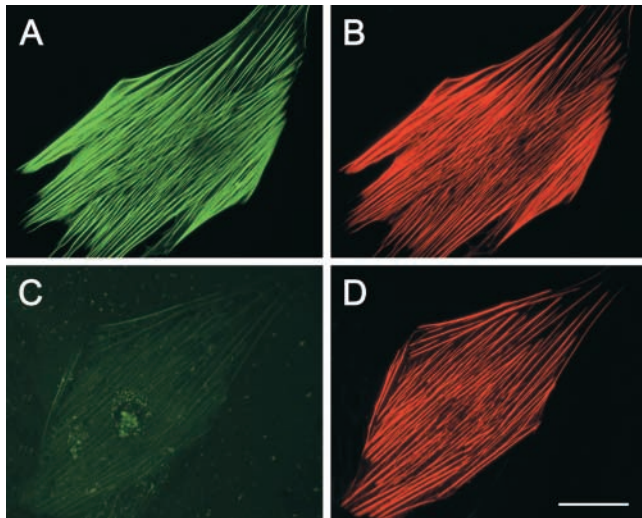
Increased synthesis of extracellular matrix proteins is a hallmark of myofibroblast activity (Serini and Gabbiani, 1999). To establish whether SMA-FP influences collagen production, we incubated cultured LFs up to 5 d in medium containing FPs at the concentration of 3–5  $\mu$ g/ml. At the end of this period, LFs maintained a shape similar to that of untreated controls (Fig. 5). When cells were double stained with  $\alpha$ -SMA antibody and phalloidin,  $\alpha$ -SMA stain was practically abolished (Fig. 5 C), whereas phalloidin distribution remained similar to that of control LFs (Fig. 5, B and D). Type I collagen mRNA expression evaluated by Northern blot started to decrease on the third day at all FP concentrations and was clearly lower than controls on the fifth day (Fig. 6). Collagen mRNA changes were paralleled by a decrease of  $\alpha$ -SMA mRNA (Fig. 6). These results suggest that the SMA-FP action on collagen and  $\alpha$ -SMA mRNA expression is exerted indirectly, for example through the decrease of fibroblast-generated tension.

During the healing of a rat open wound, the level of  $\alpha$ -SMA expressed de novo by myofibroblasts reaches a maximum in 9–10-d-old granulation tissue (Darby et al., 1990; Hinz et al., 2001b). Strips cut from this tissue exert significant isometric tension ( $130 \pm 23 \mu$ N) when stimulated with SM agonists such as endothelin (ET)-1 (Majno et al., 1971; Appleton et al., 1992; Hinz et al., 2001b). SMA-FP (0.5 mg/ml) reversibly reduced the contractile activity of granulation tissue strips to 36% compared with untreated



**Figure 4. SMA-FP inhibits LF-mediated contraction of collagen lattices.** Attached collagen lattices were treated with FPs for 30 min and released; their diameter, measured after another 30 min, was normalized to the diameter before release (equals % contraction). Compared with untreated control lattices, (ct) SKA-FP (SK) has no effect on lattice contraction, whereas SMA-FP (SM) reduces contraction dose dependently; washing out SMA-FP before release (W) reverses this effect. \* $p \leq 0.01$  and \*\* $p \leq 0.001$  compared with control.

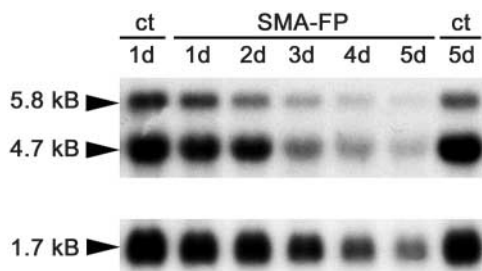




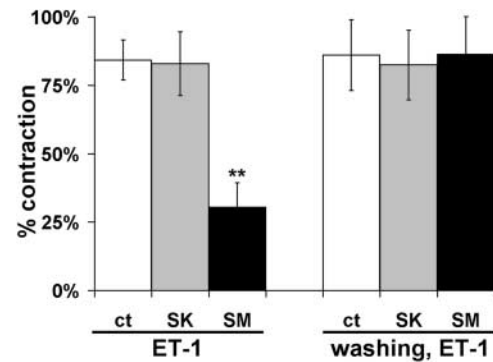
**Figure 5. Long term effect of SMA-FP on LF morphology and  $\alpha$ -SMA immunostaining.** LFs grown for 5 d in enriched serum-free medium exhibit a highly organized stress fiber apparatus that stains for  $\alpha$ -SMA (A, green) and F-actin (B, phalloidin red). Administration of SMA-FP twice a day for 5 d almost completely abolishes staining for  $\alpha$ -SMA (C) but does not change cell morphology and F-actin distribution (D). Bar, 50  $\mu$ m.

control strips or strips treated with the same concentration of SKA-FP (Fig. 7). Thus, SMA-FP has the capacity of inhibiting pharmacologically stimulated granulation tissue myofibroblast contraction; the effective dose was approximately two orders of magnitude higher compared with in vitro experiments, possibly due to the absorption of the FP to the extracellular matrix.

To investigate the effect of SMA-FP on granulation tissue contraction in vivo and to avoid as much as possible interference with epithelialization, we have selected the model of wounds splinted by means of a plastic frame (Abercrombie et al., 1960; Hinz et al., 2001b). Compared with normal healing wounds, the splinting technique increases the expression of  $\alpha$ -SMA by wound fibroblasts (Hinz et al., 2001b). The mechanical prevention of wound contraction accumulates tensile force that results in rapid wound area re-



**Figure 6. SMA-FP decreases mRNA expression of collagen I and actin in cultured myofibroblasts.** After treating cultured LFs with SMA-FP (3  $\mu$ g/ml) for 1–5 d, Northern blot analysis shows a time-dependent decrease of collagen I mRNA, detected in two bands at 4.7 and 5.8 kb, and of  $\alpha$ -SMA, detected at 1.7 kb. In both cases, the decrease is visible on the third day. Control LFs (ct) do not show changes in collagen I and  $\alpha$ -SMA mRNA after similar incubation times.

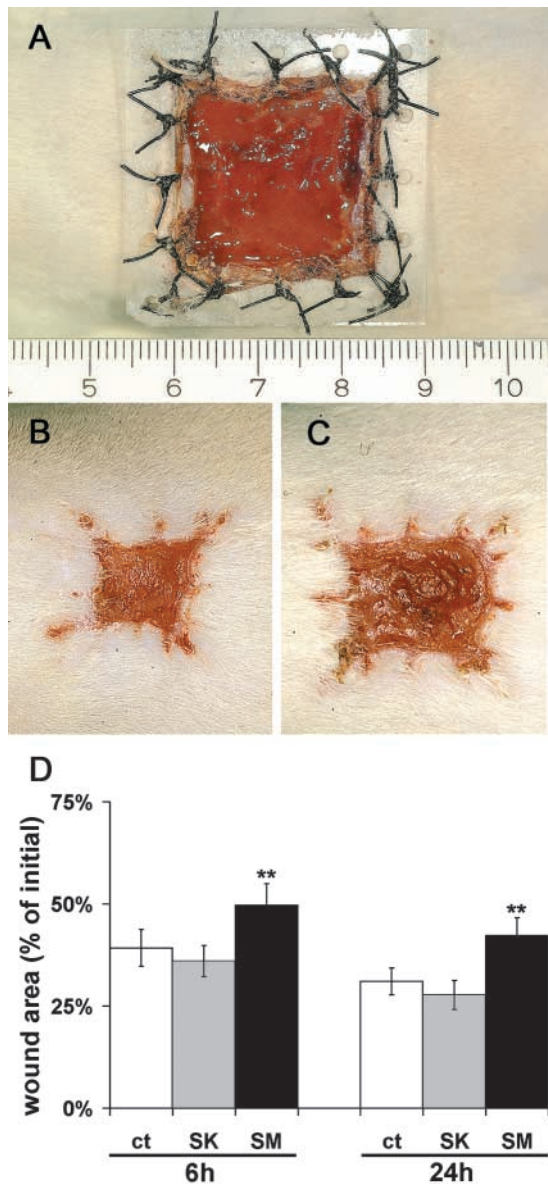


**Figure 7. SMA-FP inhibits granulation tissue strip contraction.** Strips from 9-d-old granulation tissue were stimulated with ET-1, returned to resting tension by washing for 120 min, then treated with FPs for 60 min or left untreated and stimulated a second time with ET-1. Reversibility was tested by washing 120 min and stimulating with ET-1 a third time. Treatment with SKA-FP (SK) does not change contraction compared with untreated strips (ct). SMA-FP (SM) significantly reduces strip contraction; washing out SMA-FP restores control contraction levels.  $**p \leq 0.001$  compared with control.

duction when the frame is removed. In our experiments, the frame was removed 10 d after wounding, and there was a reduction to 30% of the initial surface after 24 h (Fig. 8, A and B). The splinted wounds were treated with carrier gel alone and carrier gel containing either SKA-FP or SMA-FP once on the eighth and ninth day and just after the removal of the frame on the tenth day. 6 h after frame removal, the area of control wounds and wounds treated with SKA-FP was reduced to  $\sim 37\%$  compared with the initial wound area; application of SMA-FP significantly reduced this rapid contraction (50% of the initial wound area) (Fig. 8, C and D). After 24 h, control wound areas were further reduced to 31% of the initial wound area, whereas SMA-FP-treated wounds exhibited significantly larger areas (42%).

## Discussion

Our results show that the intracellular delivery of the  $\alpha$ -SMA NH<sub>2</sub>-terminal peptide Ac-EEED, a sequence conserved in mammalian species (Vandekerckhove and Weber, 1978), decreases specifically the tension exerted by myofibroblasts in vitro and reduces granulation tissue contraction in vivo. They support the hypothesis that  $\alpha$ -SMA through this sequence plays a role in the production of tension by myofibroblastic cells. They also support the possibility that actin isoforms exert specific activities (Chaponnier et al., 1995; Khaitlina, 2001). The selective disappearance of  $\beta$ -CA staining from stress fibers and the formation of protrusions observed in these cells after administration of  $\beta$ -CA-FP further reinforce this assumption and suggest that the NH<sub>2</sub>-terminal sequence of actin isoforms plays an important role in determining their function. Our results showing a selective disappearance of  $\alpha$ -SMA after the administration of SMA-FP and of  $\beta$ -CA after administration of  $\beta$ -CA-FP support previous findings reporting actin isoform segregation within the cytoplasm (DeNofrio et al., 1989; Gimona et al., 1994) and, albeit indirectly, the suggestion that stress fibers may contain actin filaments composed of a single isoform (for review see Gun-



**Figure 8. SMA-FP reduces in vivo wound contraction.** (A) A representative full thickness wound on the rat dorsal region was subjected to mechanical tension by splinting; the frame was left in place for 10 d. The scab was removed 8 d after wounding, and wound tissue was treated with FPs in carrier gel or with carrier gel only. Treatment was repeated on the ninth and tenth day after wounding. (B) 24 h after splint removal, the wound treated with SKA-FP exhibits an important surface reduction comparable to that of untreated controls. (C) The wound treated with SMA-FP exhibits a significantly less important reduction. (D) Wound area was measured 6 and 24 h after splint removal and normalized to the initial wound area. Mean values were calculated using 20 animals per experimental condition. ct, carrier gel only; SK, SKA-FP; SM, SMA-FP. \*\* $p \leq 0.001$  compared with control.

ning et al., 1998). We have reported previously that microinjection of  $\alpha$ -SMA antibody in myofibroblasts results in stress fiber disappearance (Skalli et al., 1990); however, the morphological results and the kinetics of changes were clearly different from the results described here.

The mechanism of collagen mRNA expression decrease in SMA-FP-treated cells is presently not clear. It is conceivable

that tension reduction after the application of SMA-FP is in turn responsible for the decrease of collagen and  $\alpha$ -SMA mRNA expression that takes place starting at 3 d after SMA-FP application. The expression of  $\alpha$ -SMA in myofibroblasts has been shown recently to decrease with decreasing tension of (a) collagen substrate in culture (Arora et al., 1999) and (b) rat granulation tissue (Hinz et al., 2001b). Based on these findings, we suggest that SMA-FP can exert beneficial effects on excessive wound contraction and fibrocontractive diseases for which no efficient pharmacological therapy exists at present.

The mechanism of tension generation by myofibroblasts is not established. It is accepted that it depends on development and organization of stress fibers (Burridge, 1981; Tomasek et al., 1992; Katoh et al., 1998). More and more evidence is accumulating in favor of the possibility that the incorporation of  $\alpha$ -SMA in myofibroblast stress fibers is essential for controlling the level of force production by these cells (Arora and McCulloch, 1994; Hinz et al., 2001a). It is noteworthy that the inhibition of myofibroblast contraction in vitro and in vivo by SMA-FP was always partial. This is in accordance with the possibility that stress fibers containing only cytoplasmic actins exert a contractile activity and that incorporation of  $\alpha$ -SMA in stress fibers increases importantly their contractile force (Hinz et al., 2001a).

The expression of  $\alpha$ -SMA and collagen and that of other extracellular matrix components, such as ED-A fibronectin, is stimulated coordinately by TGF- $\beta$ , a key factor in granulation tissue evolution and development of fibrocontractive diseases (Sporn and Roberts, 1992; Desmoulière et al., 1993; Rønnov-Jessen and Petersen, 1993; Gaudie et al., 1999); it will be important to study the effect of SMA-FP on the expression and/or activation of this growth factor. Further work is also required to (a) establish whether SMA-FP acts through its effect on  $\alpha$ -SMA polymerization (Chaponnier et al., 1995) and/or through a more direct influence on actin-myosin-dependent force generation and (b) identify an hypothetical SMA-FP partner at the level of the myofibroblast contractile apparatus.

In addition to providing new insight into cellular mechanisms of contraction, our results present a strategy for (a) exploring the role of actin isoforms in cell and tissue activities, (b) better understanding the mechanisms of stress fiber function and of granulation tissue contraction, and (c) developing an hitherto unexplored approach to control tissue remodeling during normal and pathological wound healing and fibrocontractive diseases.

## Materials and methods

### Fusion peptides

SMA-FP, SKA-FP, and  $\beta$ -CA-FP, containing Ac-EEED, Ac-DEDE, and Ac-DDDIA, respectively, at the NH<sub>2</sub> terminus of the cell-penetrating vector pAntp-Pro50 (Derossi et al., 1994), were synthesized to a purity of 95% (UCB Bioproducts); this vector was chosen for its efficiency in cytoplasmic delivery (Derossi et al., 1994). The two first FPs were also tagged with Rh B, covalently linked through a tetra-Glycine arm with a lysine in position 5 (UCB Bioproducts). This did not change their activity. After preliminary experiments, SMA-, SKA-, SMA-Rh-, and SKA-Rh-FP were added at the following concentrations if not stated otherwise: 3–10  $\mu$ g/ml to cultured cells on plastic and silicone substrates, 5–250  $\mu$ g/ml to cells in collagen lattices, 500  $\mu$ g/ml to excised granulation tissue strips, and 500  $\mu$ g/ml carrier gel (Lutrol® F-127; BASF AG) in the treatment of in vivo wounds (see below for experimental details).  $\beta$ -CA-FP was used at concentrations of 3–500  $\mu$ g/ml.



### In vitro experiments

LFs were isolated and cultured as described previously (Desmoulière et al., 1992) and cultured for 4 d on 60-mm culture dishes (Nunc and Life Technologies) in MEM plus 10% FCS. Experiments were performed in serum-free MEM to avoid FP precipitation. LFs were incubated with Rh-tagged FPs, fixed with methanol, and stained for  $\alpha$ -SMA (anti- $\alpha$ SM-1, IgG2a monoclonal antibody) (Skalli et al., 1986) and  $\beta$ -CA ( $\beta$ 74, rbAb) (Yao et al., 1995) followed by the secondary antibodies goat anti-mouse FITC-conjugated and goat anti-rabbit aminomethylcoumarin acetate- or Rh-conjugated (Jackson ImmunoResearch Laboratories). Rh-phalloidin (Molecular Probes) staining was performed after fixation with 3% paraformaldehyde/PBS and permeabilization with 0.2% Triton X-100/PBS. Mounted samples were observed with a 40 $\times$  oil immersion objective on an Axiophot microscope (ZEISS). Images were acquired with a digital color camera and corresponding software (Axiocam; ZEISS). All images were processed for printing with the use of Adobe Photoshop<sup>®</sup>.

To visualize the contractile activity of single cells, LFs were cultured for 4 d in the presence of FCS on deformable silicone substrates (Harris et al., 1980), which were produced in order to obtain an optimal resistance, as previously described (Hinz et al., 2001a). Image sequences were obtained with an inverted microscope (Axiovert 135; ZEISS) equipped with a 63 $\times$  objective B/W camera (BC-2; AVT Horn), frame grabber (Meteor PCI; Matrox Electronic Systems Ltd.), and KS400 software (ZEISS). FPs were added after 60 min control recording in serum-free MEM (see above) and subsequently removed by repeated washing in order to test reversibility.

Attached collagen lattices (Grinnell, 1994) were produced and mixed with LFs ( $2.5 \times 10^5$ /ml) as described previously (Hinz et al., 2001a) and cultured for 5 d in MEM plus 10% FCS. Lattices were then treated with FPs, released, and the measured diameter after 30 min was normalized to the diameter before release (equals % contraction). Reversibility was tested by washing the treated lattices before release.

### RNA extraction and Northern blotting

LFs were grown to 80% confluence in 10-cm cell culture dishes in MEM plus 10% FCS; cells were then cultured up to 5 d in enriched serum-free medium (Stouffer and Owens, 1992) containing FPs at the concentration of 3–5  $\mu$ g/ml; the culture medium with FPs was changed twice a day. Total RNA was isolated using Trizol reagent (Invitrogen) according to the manufacturer's protocol. Total RNA (2.5–5.0  $\mu$ g per lane) was denatured, separated by electrophoresis, and transferred onto an Electran nylon membrane (BDH Laboratory Supplies) as described previously (Serini et al., 1998). Hybridization was performed with two probes: a 1,600-bp rat  $\alpha$ 1(I) collagen cDNA, recognizing two bands at 5.8 and 4.7 kb (Genovese et al., 1984), and a 320-bp total actin cRNA (Kocher and Gabbiani, 1986), recognizing  $\alpha$ -SMA mRNA at 1.7 kb and cytoplasmic actin mRNA at 2.1 kb as described previously (Kocher and Gabbiani, 1986; Serini et al., 1998). Quantification of the signal was normalized to results obtained using GAPDH probe (Neuville et al., 1997). After hybridization, filters were washed 2  $\times$  20 min at 65°C (collagen) and 58°C (actin), respectively, in 5 $\times$  SSC and 0.1% SDS. Northern blots were then exposed on Eastman Kodak Co. X-Omat SO-282 film at –70°C.

### Animal experiments

To measure isometric tension of excised granulation tissue strips, a total of 40 female Wistar rats (200–250 g) were used. All animal procedures were performed according to Federal Veterinary Guideline and approved by the Ethical Committee of the Geneva Medical Faculty. Full thickness 25  $\times$  25-mm wounds were made in the middle of the dorsum (Hinz et al., 2001b). Rats were killed by CO<sub>2</sub> anesthesia 9 d after wounding, and the tissue was dissected and cut into strips of 5  $\times$  10 mm. Strips were prestressed in an organ bath on the levers of an isometric force displacement transducer (FT03; Grass Instrument Co.) (Hinz et al., 2001b), stimulated with ET-1 (Bachem AG)  $10^{-7}$  M, returned to resting tension by washing for 120 min, and then treated with FPs for 60 min or left untreated and stimulated a second time with ET-1. Reversibility was tested by washing 120 min and stimulating with ET-1 a third time. Peak tension was measured  $\sim$ 30 min after each stimulation, and mean values were calculated from three tissue strips per animal and 10 animals per experimental condition as described previously (Hinz et al., 2001b).

To test in vivo wound contraction, a total of 80 female Wistar rats (200–250 g) were used. Full thickness 25  $\times$  25-mm wounds (see above) were subjected to mechanical tension by splinting, that is, fixing their edges to a plastic frame by means of surgical thread; the frame was left in place for 10 d (Hinz et al., 2001b). The scab of splinted wounds was removed 8 d after wounding, and wound tissue was treated with FPs in 1 ml of carrier gel (Lutrol<sup>®</sup> F-127; BASF AG) or with carrier gel only and was covered with a

transparent film (OpSite<sup>™</sup>; Smith & Nephew Medical Ltd.). Treatment was repeated on the ninth and tenth day after wounding immediately after removal of the splint. Wound area was measured immediately before removing the plastic frame and after 6 and 24 h. Wound contraction was calculated as area reduction normalized to the initial wound size. Mean values were calculated using 20 animals per experimental condition.

### Statistical analysis

Quantitative results are presented as mean values  $\pm$  SD. Differences between mean values were tested by means of a two-tailed heteroscedastic Student's *t* test and were considered to be statistically significant at values of  $*p \leq 0.01$  and  $**p \leq 0.001$ .

### Online supplemental material

The video (available at <http://www.jcb.org/cgi/content/full/jcb.200201049/DC1>) demonstrates the effect of the SMA-FP on the wrinkling capacity of LFs on silicone substrates. The sequence starts with an untreated LF that has already been observed for 60 min, producing wrinkles on a deformable silicone substrate without visible changes. After treatment with SMA-FP, wrinkles gradually disappear within 30 min. After two washing steps, the LF contracts again restoring wrinkles completely after 60 min.

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