

A Function for Filamentous α -Smooth Muscle Actin: Retardation of Motility in Fibroblasts

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Abstract. Actins are known to comprise six mammalian isoforms of which β - and γ -nonmuscle actins are present in all cells, whereas α -smooth muscle (α -sm) actin is normally restricted to cells of the smooth muscle lineages. α -Sm actin has been found also to be expressed transiently in certain nonmuscle cells, in particular fibroblasts, which are referred to as myofibroblasts. The functional significance of α -sm actin in fibroblasts is unknown. However, myofibroblasts appear to play a prominent role in stromal reaction in breast cancer, at the site of wound repair, and in fibrotic reactions. Here, we show that the presence of α -sm actin is a signal for retardation of migratory behavior in fibroblasts. Comparison in a migration assay of fibroblast cell strains with and without α -sm actin revealed migratory restraint in α -sm actin-positive fibroblasts. Electroporation of monoclonal antibody (mAb) 1A4, which recognizes specifically the NH₂-terminal Ac-EEED sequence of α -sm actin, significantly in-

creased the frequency of migrating cells over that obtained with an unrelated antibody or a mAb against β -actin. Time-lapse video microscopy revealed migratory rates of 4.8 and 3.0 μ m/h, respectively. To knock out the α -sm actin protein, several antisense phosphorothioate oligodeoxynucleotide (ODNs) were tested. One of these, 3'UT1, which is complementary to a highly evolutionary conserved 3' untranslated (3'UT) sequence of α -sm actin mRNA, was found to block α -sm actin synthesis completely without affecting the synthesis of any other proteins as analyzed by two-dimensional gel electrophoresis. Targeting by antisense 3'UT1 significantly increased motility compared with the corresponding sense ODN. α -Sm actin inhibition also led to the formation of less prominent focal adhesions as revealed by immunofluorescence staining against vinculin, talin, and β 1-integrin. We propose that an important function of filamentous α -sm actin is to immobilize the cells.

ACTIN has been implicated as the major cytoskeletal element in cellular locomotion. In deciphering the role of actin for cellular movement, attention has been focused mainly on two different cytoskeletal organelles, the strapped-down stress fibers more or less spanning the cell body and the fine meshwork of actin in protrusive lamellipodia (13, 62). Due to the concurrent expression of both structures in fibroblasts, there is a long tradition for the use of these cells in studies of cellular motility. Fibroblasts have been considered to contain two actin isoforms only, i.e., β - and γ -nonmuscle actin, as determined by biochemical analysis of the NH₂-terminal tryptic peptide. Whereas these isoforms are ubiquitously expressed by all eukaryotic cells, γ -smooth muscle(sm),¹ α -cardiac, α -skeletal, and α -sm actin have been considered tissue specific (22, 68, 69). Recently, however, a new dimension has emerged in the field of fibroblast cell biology. It has been realized that fibroblasts dramatically turn on one of

the muscle-specific isoforms of actin, α -sm actin, in a number of pathological settings affecting the interstitial stroma (7, 11, 19, 38, 50, 51, 53, 55). Moreover, most fibroblasts kept under conventional culture conditions with varying amounts of serum in the medium appear to be in a state of continuous activation in terms of α -sm actin expression (15, 48, 52, 63). The activity in serum and in interstitial tissue responsible for this activation of fibroblasts has been determined to be that of TGF- β (14, 49).

The functional significance of the ectopic expression of α -sm actin in fibroblasts has remained a mystery (9). However, a number of observations suggest that it may modulate the motor function of actin in general. First of all, based mainly on studies of pericytes, it appears that the actin isoforms are differentially compartmentalized in the two main organelles of actin. Thus, whereas α -sm actin is rapidly and selectively incorporated into stress fibers, β -actin readily accumulates in the leading lamellipodia (13, 26). Second, in vascular smooth muscle cells normally

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1. Abbreviations used in this paper: DME-F12, DME-Ham's F12; ODN, oligodeoxynucleotide; sm, smooth muscle.

expressing all three isoforms, the α isoform is downregulated as the cells translocate during atherosclerosis (6). Conversely, α -sm actin is highly expressed in fibroblasts when cell-substratum traction may be more important than motility such as during wound contraction and in chronic fibrotic conditions (12, 14, 19, 29, 54).

An obvious strategy for analyzing α -sm actin function would seem to be either complete or partial gene knock-out by antisense transfection, or expression of the full-length cDNA clone for α -sm actin. The first option would require cell lines with universal and homogenous expression, as is the case, for instance, for vinculin (21). However, this has been an elusive goal as far as α -sm actin-expressing cells are concerned. Thus, by cloning at random, it has been exhaustively demonstrated that no two fibroblast clones are identical when it comes to the level of α -sm actin expression (15). This prevents the generation of control clones with stable expression. As for the second option, the expression of actin proteins in nonmuscle cells has been further hampered by induction of lethal or grossly aberrant phenotypes (71). Even if a full cDNA clone were available, no α -sm actin-negative, clonable fibroblast cell lines have so far been available. Therefore, expression of α -sm actin in stable, nonexpressing cells would have necessitated other cell types to be used, leading to a completely different cytoskeletal structure. In addition, it would be preferable if the function of this protein could be assessed in its natural environment and not as an overexpressed protein.

We have previously approached the question of obtaining α -sm actin-positive fibroblasts by use of a short-term culture assay of human breast fibroblasts on collagen-coated plastic (49, 52). In this assay, we readily generate cell strains with up to 90% of cells expressing strong α -sm actin. We are also able to control the activated phenotype under strictly reproducible serum-free conditions by use of TGF- β and adhesion-modified plastic substratum (49). In this study we take advantage of these recently developed culture technologies as well as novel fibroblast cell strains that essentially fail to express α -sm actin. We show that the α -sm actin-expressing fibroblasts migrate slower than the nonexpressing cells. By tagging the α -sm actin protein at the isoform-specific NH₂-terminal end (68) with an electroporated α -sm actin-specific antibody, we further narrow the cause-effect relation between α -sm actin expression and migratory behavior. Finally, we use antisense ODNs against the isoform-specific 3'UT region of the mRNA (32, 47) and show that α -sm actin synthesis is completely and specifically abolished and migration is increased accordingly.

Materials and Methods

α -Sm Actin-positive and -negative Fibroblasts

Normal breast tissue was obtained from 77 reduction mammoplasties performed for cosmetic reasons. The tissue was cut, collagenase-digested in DME-Ham's F12 (DME-F12) with 2 mM glutamine (Sigma Chem. Co., St. Louis, MO), 50 μ g gentamicin/ml (Garamycin; Schering, Kenilworth, NJ), and essentially pure cell strains of α -sm actin-positive fibroblasts were generated by induction with FCS as previously described (52). These fibroblasts were used in passages 2 and 3. In cultures from a few random biopsies (F359, F498, and F514), fibroblasts without α -sm actin spontane-

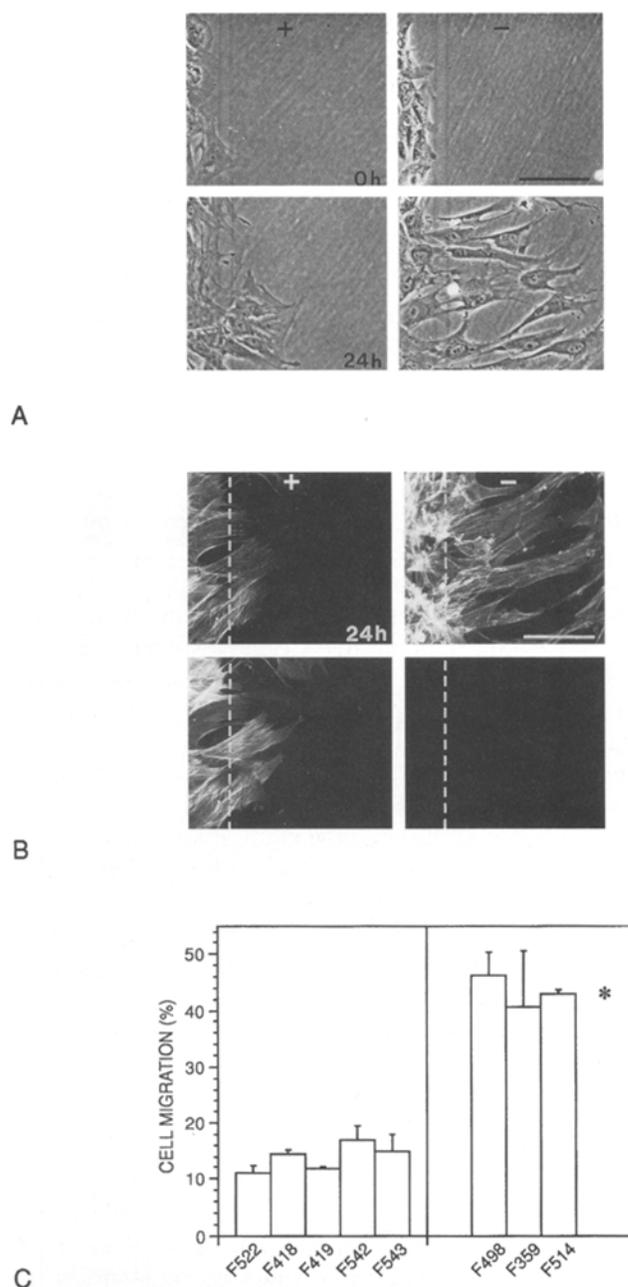


Figure 1. Fibroblast motility correlates with isoactin phenotype. (A) Phase contrast micrographs of the α -sm actin-positive (left; +) and -negative (right; -) cell strains in the migration assay photographed at time zero (top) and after 24 h (bottom). The cell densities were essentially identical behind the starting lines, but the α -sm actin-negative cells migrated in much higher numbers and for longer distances compared with the α -sm actin-positive cells. (B) Characterization of isoactin expression in fibroblast cell strains after 24 h of migration. The dotted line indicates the starting line. The cells were double stained by immunofluorescence for filamentous actin by FITC-phalloidin (top) and α -sm actin by mAb 1A4 (bottom). The general filamentous actin phenotype is almost identical in the two cell strains, but one of them does not express α -sm actin. (C) Diagram showing the percentages of cell migration at $t = 24$ h of five α -sm actin-positive (left) and three α -sm actin-negative (right) cell strains. The use of an unpaired Student's t test with two tails indicates that the difference is significant ($*P < 0.01$). Magnification, 150; Bars: (A and B) 100 μ m.

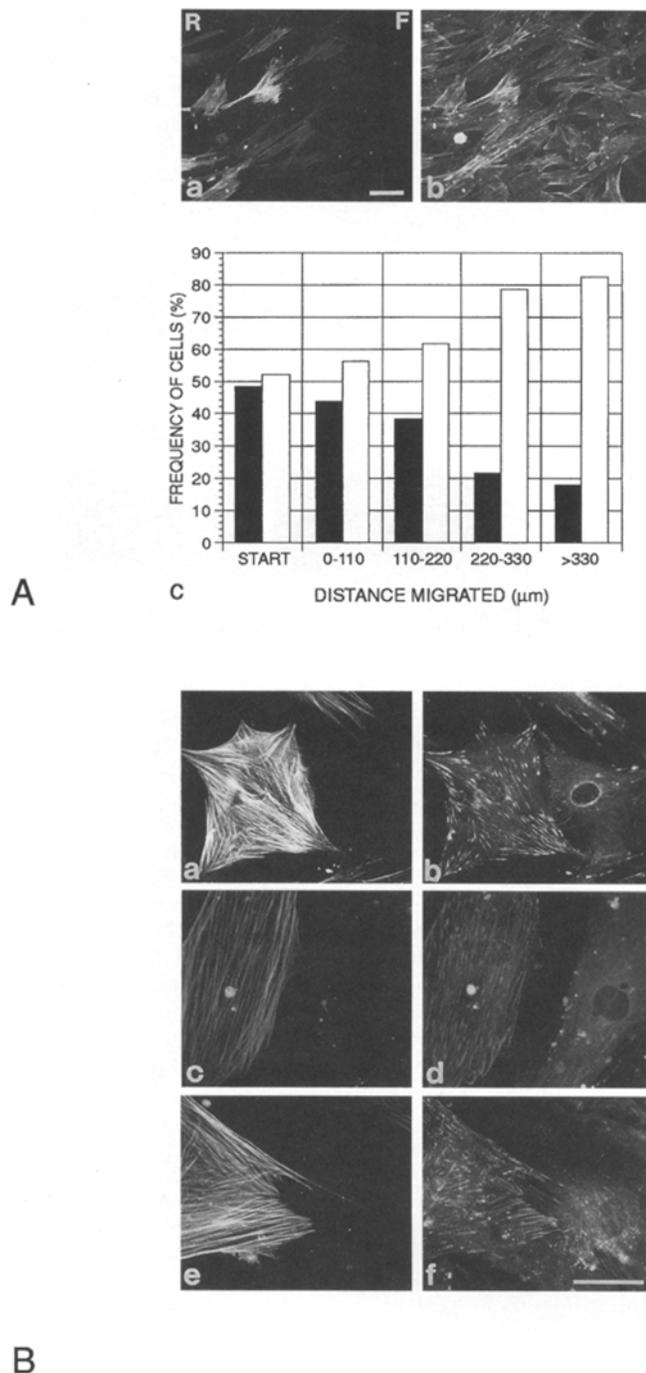


Figure 2. The difference in migratory behavior between α -sm actin-positive and -negative cells correlates with cell-matrix interactions. (A) Heterotypic cell interactions were assayed between roughly equal numbers of α -sm actin-positive and -negative fibroblasts lined up next to each other at a starting line. After free migration for 48 h, cells were double stained for α -sm actin (a) and general actin (b). α -Sm actin-positive cells as quantified by immunoperoxidase staining travel a much shorter distance (mean of relative percentage) (c). Closed bars, α -sm actin-positive cells; open bars, α -sm actin-negative cells. Magnification: 60; R, rear; F, front. (B) Cell-matrix interactions were expressed in terms of focal contact formation in the mixed field of α -sm actin-positive and -negative fibroblasts. Cells were double stained for α -sm actin (a, c, and e) and vinculin (b), talin (d), or β 1-integrin (f). The focal contact phenotype strongly corroborates the less motile be-

havior, i.e., prominent focal contacts are scattered throughout the entire contact area of the α -sm actin-positive cells. Magnification: 250; Bars: (A) 100 μm ; (B) 50 μm .

ously predominated at an early stage. Similar to the α -sm actin-positive cell strains mentioned above, these essentially α -sm actin-negative fibroblasts grew in DME-F12 with 20% FCS.

A 50-50 mixture cell strain, a subline of F359, was generated by cultivation until the third passage in CDM3 (45) supplemented with 20% FCS, and then shifted to DME-F12 with 20% FCS to induce α -sm actin and used in the sixth passage. The subline essentially maintained its actin expression pattern irrespective of cell culture density and migratory status. All migration experiments were performed in DME-F12 with 20% FCS.

For depletion of α -sm actin by 3'UT1 antisense (see below), α -sm actin-negative fibroblasts were explanted under controlled serum-free conditions as previously described (49). They were next primed with 20% FCS for 48 h, electroporated with ODNs as described below, and then plated in DME-F12 with either 20% FCS for 24 h or TGF- β 1 (100 pg/ml; Sigma Chemical Co.) for 72 h in the presence of 1 μM ODN to induce α -sm actin as previously described (49, 52).

Migration Assay

A bar was placed in a collagen-coated (8 $\mu\text{m}/\text{cm}^2$ Vitrogen 100; Collagen Corporation, Palo Alto, CA) six-well dish (Nunc, Roskilde, Denmark) on overlapping pieces of sterile thermanox coverslips (Miles Laboratories Inc., Naperville, IL), and the thermanox was removed, leaving a small space between the bar and the well, and allowing placement of a line of dots or a thin continuous line made with a glass rod up against each side of the bar. The bar was pressed down, and free passage of medium was allowed through channels in the bar perpendicular to its orientation, counteracting leak-in of cells under the bar by the capillary effect. 2×10^5 cells were plated per well from essentially nondividing, confluent cultures. Thus, cell division did not start to any significant degree during the experiment. The demarcation did not restrain migrating cells. Migration was quantified at 37°C with a grid reticule (1,100 \times 1,100 μm) in the eye piece.

The percentage of migrating cells in each experiment was obtained as follows. The mean number of cells in 36 fields of $110 \times 1,100 \mu\text{m}^2$ perpendicular to and immediately in front of the starting line was calculated (migrating cells) and divided by the mean number of cells in six consecutive fields of $110 \times 3,300 \mu\text{m}^2$ counted at a predefined distance behind the starting line (cell density). Both the number of migrating cells and the cell density behind the line were determined for each time point. The cell density behind the line remained essentially unchanged during the experiment, and the mean cell densities within each category of experiments ($n = 8$) showed little variation and was not significantly different. Unless otherwise stated, the values are given as mean \pm SEM. Statistics were performed using an unpaired Student's t test with two tails.

Time Lapse Video Microscopy

To study migration at the individual cell level, GIPS Locomotory Area Recording (GIPSLAR; Image House, Copenhagen, Denmark) software was used. Cultures were placed on an inverted phase-contrast microscope equipped with a heating device equilibrated to maintain 37°C. In the period of 24–35 h after electroporation, 12 video images were recorded at 1-h intervals to an optical disc recorder. Migration tracks were generated by marking the position of the nucleus of individual cells on each image. The net migratory speed (“velocity straight line”) was calculated by GIPS Track Area Analysis (GIPSTRK; Image House) software based on the straight line distance between the starting and ending points divided by hours of observation. Values are given as mean \pm SEM.

Electroporation

For electroporation, a gene pulser apparatus (Bio-Rad Laboratories, Richmond, CA; purchased from Bie & Berntsen, Rødovre, Denmark) was used. The optimal conditions for electroporation were determined by comparing the prevailing settings published by Wilson et al. (73), Lukas et al. (36), and Glogauer and McCulloch (25), including an additional setting of 25 μF and up to 1.7 kV. Fibroblasts were electroporated at 0–4°C at an electrode distance of 0.4 cm and 0.25 kV (field strength 625 V/cm) at 500 μF , or for some experiments, 960 μF (25). The cells were trypsinized,

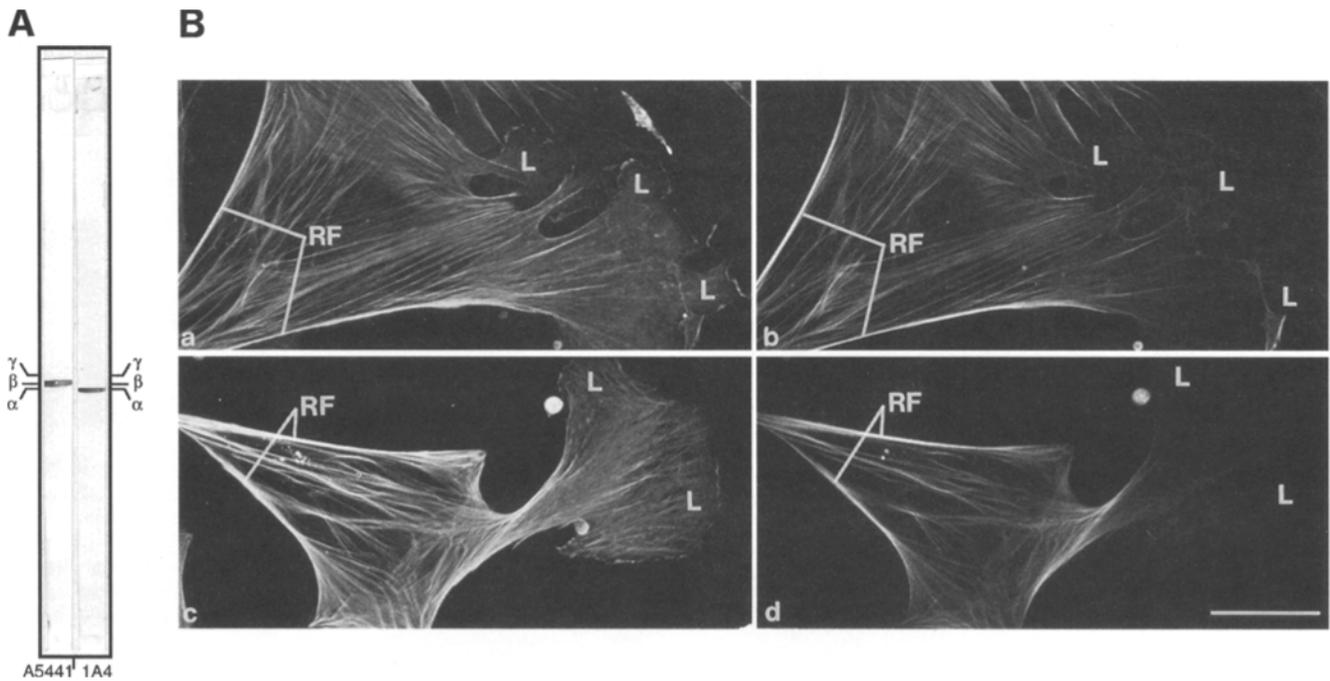


Figure 3. Tagging filamentous α -sm actin with an electroporated mAb in α -sm actin-positive fibroblasts overcomes the migratory restraint. (A) Isoelectric focusing and immunoblotting shows the specificity of the β -actin (A5441), and α -sm actin (1A4)-specific antibodies used for electroporation. (B) Double-immunofluorescence of α -sm actin-positive fibroblasts with mAbs against β -actin (a and c) and α -sm actin (b and d). Whereas β -actin is present in both stress fibers and lamellipodia (L), α -sm actin predominates in stress fibers, in particular retraction fibers (RF). (C) Immunofluorescence tracing of antibodies electroporated into α -sm actin-positive fibroblasts. Cells were electroporated with mAb against β -actin (a), mAb against α -sm actin (b), or no antibody (c) and stained 16 h after plating by Texas red-conjugated secondary antibodies. Whereas cells electroporated with anti- β -actin exhibited staining of stress fibers, cells electroporated with anti- α -sm actin exhibited predominantly cytoplasmic staining. (D) Time course of the mean cell migration comprising eight different experiments with cells electroporated with mAb 1A4 against α -sm actin (\circ), an unrelated antibody, Z259 (\bullet), or β -actin-specific mAb (\blacksquare) at the same concentration. The use of an unpaired Student's *t* test with two tails indicates that the difference is significant (mean \pm SEM, $n = 8$; $*P < 0.05$). (E) Time lapse video microscopy showing the difference in mean migration in microns per hour based on six experiments, each including ~ 75 cells electroporated with either an unrelated antibody (control) or an mAb against α -sm actin (1A4). An unpaired Student's *t* test with two tails indicates that the difference is significant ($*P < 0.05$). Magnifications: (B) 430; (C) 400. Bars: (B) 50 μ m; (C) 20 μ m.

counted, centrifuged, and resuspended in 400 μ l electroporation buffer (73) with antibody (1 mg/ml) or ODN (10 μ M), electroporated by a single discharge, resuspended in DME-F12 with 5% FCS and plated, and allowed to migrate in DME-F12 with 20% FCS. All antibodies used for electroporation were dialyzed against electroporation buffer to remove azide before experimentation. Antibodies were 1A4 (A2547; Sigma Chemical Co.); an mAb specifically recognizing α -sm actin (60), and rabbit anti-mouse immunoglobulins, Z259 (Dako, Glostrup, Denmark), or an mAb recognizing β -nonmuscle actin (A5441; Sigma Chemical Co. [23] as controls. Both 1A4 and A5441 are monospecific for their respective isoforms. 1A4 binds to the first four amino acids Ac-EEED in α -sm actin, and A5441 binds to the first five amino acids Ac-DDDIA in β -actin (9, 23).

Antisense/Sense ODNs

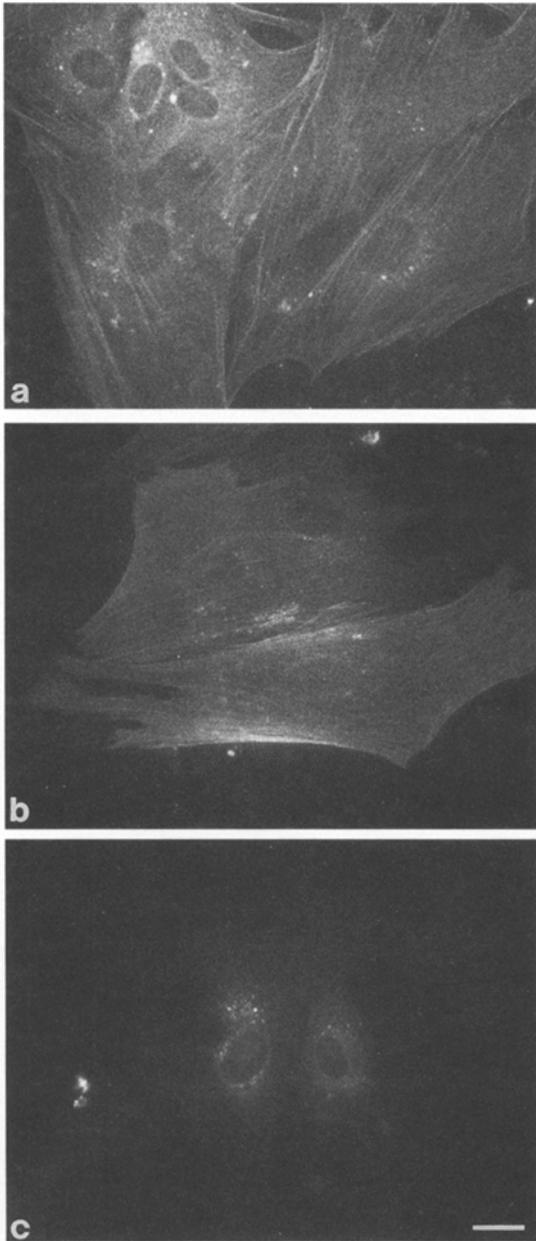
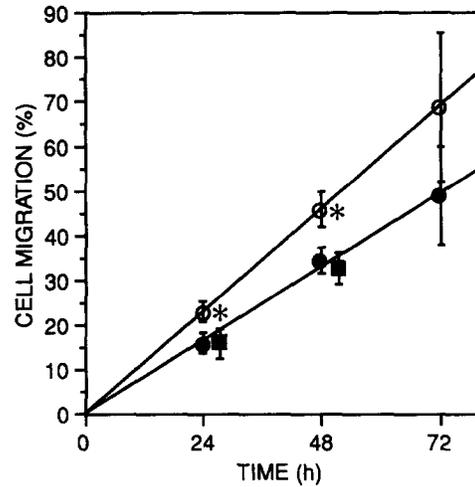
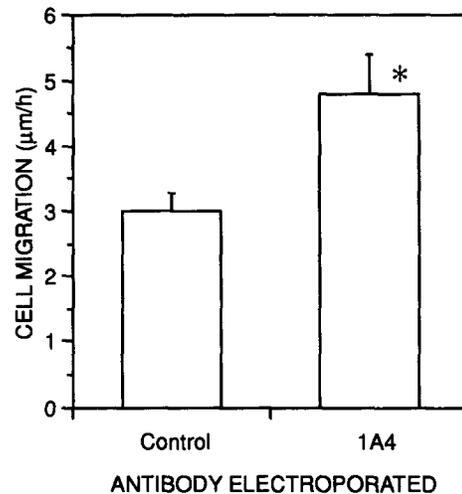
To target α -sm actin mRNA, we designed several HPLC-purified phosphorothioate ODNs based on the known nucleotide sequence of human α -sm actin cDNAs (EMBL accession No. x13839 [32, 32a]). Phosphorothioate-modified ODNs were used as they have been shown by others to be more stable and effective than unmodified ODNs (74). To ensure optimal effect of ODNs and to be able to directly compare the experiments with antibody experiments, ODNs were electroporated as described above.

mRNA coding for the NH_2 -terminal decapeptide of α -sm actin was targeted by 5' AGTGCTGTCCTTCTTCACACATA 3' (4, 14; here termed 5'CO antisense). Controls were 5' TATGTGTGAAGAAGAGGACAGCACT 3' (5'CO sense) or 5' AATGGTGTGCTTTTGTCCCA-GATA 3' (noncomplementary [74]). To target sequences of the 3' untranslated region of α -sm actin mRNA (32), six antisense phosphorothioate ODNs were tested: 5' CACAGTTGTGTGCTAGAGAC 3' (3'UT1); 5'

TAACGAGTCAGAGCTTTGGC 3' (3'UT2); 5' TCAACCTAACAAATGGTATC 3' (3'UT3); 5' AGGTAATGTGATTCTACCCT 3' (3'UT4); and 5' GAAGTGCAGCTCCCCACTC 3' (3'UT5); and 5' AGAGAGGAGCAGGAAAGTGT 3' (3'UT6). Control was 5' GTCTCTAGCACAACTGTG 3' (3'UT1 sense). ODNs were synthesized by DNA technology (Aarhus, Denmark) and kept at -20°C until use.

Immunocytochemistry

The actin status was investigated by immunofluorescence after two different fixation protocols. In double-labeling experiments comprising decoration of F-actin with the general actin probe FITC-phalloidin (P-5282; Sigma Chemical Co.), combined with staining for α -sm actin, cultures were fixed for 2 min at room temperature in glutaraldehyde/Triton X-100 (0.25/0.3%) and incubated as described (49). In other double-labeling experiments with α -sm actin, cultures were fixed in methanol for 5 min at -20°C . Subsequently, cultures were incubated with 1A4 (IgG2a) against α -sm actin and one of the following IgG1 antibodies in the first sequence: β -actin-specific mAb A5441 (IgG1), antivinculin (kindly provided by Drs. Victor E. Koteliansky and Marina A. Glukhova, Laboratoire du Physiopathologie du Developpement, CNRS and École Normale Supérieure, France), anti- β 1-integrin (clone DF5; BIOHIT, Helsinki, Finland) or antitalin (clone 8d4; Sigma Chemical Co.), and goat anti-mouse IgG2a-Texas red (1080-07; Southern Biotechnology Associates, Birmingham, AL) and goat anti-mouse IgG1-FITC (1070-02; Southern Biotechnology Associates) in the second sequence. For tracing of electroporated antibodies, cells were stained at 16, 24, 48, and 72 h after electroporation with goat anti-mouse IgG1-Texas red (1070-07; Southern Biotechnology Associates) for A5441 or 1080-07 for 1A4. As negative controls served the op-

C**D****E**

posite combinations of staining and cells electroporated with no antibody. Cultures were mounted in Fluoromount-G (100, Southern Biotechnology Associates) containing 2.5 mg/ml *n*-propyl gallate (Sigma Chemical Co.) used between day 1 and 1 mo of preparation. The experimental procedure for immunoperoxidase staining and quantitation of α -sm actin-positive fibroblasts was performed as previously described (49).

Isoelectric Focusing, SDS-PAGE, Immunoblotting, and Two-dimensional Gel Electrophoresis

Cells were rinsed twice in PBS at room temperature and lysed in Laemmli buffer for SDS gels or 8 M urea buffer for IEF and two-dimensional gels to obtain whole cell lysates (51). IEF gels were loaded with equal amounts of lysate ($\sim 15 \mu\text{g}$ protein/lane as determined by the Bio-Rad protein assay [51]). Gel plates were 16×16 cm with 0.5 mm spacers; 8.24 g urea, 1.95 ml 28.38% wt/vol acrylamide (Bio-Rad Laboratories), 1.62% bisacrylamide (Bio-Rad Laboratories, 3.0 ml NP-40, 2.4 ml H_2O , 0.93 ml carrier

ampholytes, pH 5–7 (Serva Feinbiochemica, Heidelberg, Germany, purchased from Bie & Berntsen), 0.31 ml carrier ampholytes, pH 4–6 (Bio-Rad Laboratories), 15 μl 10% ammonium persulfate, and 10 μl N,N,N',N'-Tetramethyl-ethylenediamine (8, 24). SDS-polyacrylamide gels (12% running gel, 5% stacking gel) were loaded with equal amounts of lysed cells ($\sim 5 \times 10^5$ /lane) and run as previously described (51). Immunoblotting of IEF and SDS-polyacrylamide gels was carried out by transfer to Immobilon transfer membrane (Millipore; Tåstrup, Denmark) (51) followed by incubation with A5441 against β -actin and 1A4 against α -sm actin. Corresponding blots of gels loaded with the same amount of protein were stained with Coomassie blue for demonstration of total protein level (48). For two-dimensional gel electrophoresis, cells were incubated with [^{35}S]methionine, and samples were prepared and run as previously described (51). The exact position of actins was verified by comigration with purified bovine actin (A3653; Sigma Chemical Co.). Films were scanned by use of a film scanner (model JX330; Sharp Electronics, Hamburg, Germany) and processed in a 133 MHz, 1 Gb, computer using the DOS edition of PDQUESTTM, version 5.1 (Pharmacia, Birkerød, Denmark).

Results

Motility of Fibroblasts with α -sm Actin Expression

A bar was placed in the middle of a culture dish, and a thin continuous line or a line of dots was drawn with a glass rod up against the bar. Cells were then plated and the bar was removed, allowing the cells to migrate into the cell-free area. To compare the migration potential of fibroblasts with and without α -sm actin in this assay, two types of cell strains were used (Fig. 1 A). Both contained distinct stress fibers of F-actin independently of their migratory status, but only one of them expressed α -sm actin (Fig. 1 B). Under identical, essentially nongrowing conditions, the two types of cell strains showed a significant difference in migration potential (Fig. 1 C).

To distinguish whether the observed difference in migratory behavior was dependent on homotypic cell-cell interactions (66) or cell-matrix interactions, a cell line (subline of F359) constantly generating a 50–50 mixture of α -sm actin-positive and -negative fibroblasts was developed. Thus, these cells had an entirely identical passage and culture history, and no fluctuation in actin isoform expression was observed during the experimental period. As seen in Fig. 2 A, α -sm actin-positive fibroblasts were found to migrate a shorter distance even in a mixed population. Moreover, these cells had higher numbers of prominent focal contacts (often >200/cell as shown by staining for vinculin, talin, and β 1-integrin) as opposed to α -sm actin-negative cells (Fig. 2 B), providing a possible explanation for why these cells were less motile (21, 37).

Fibroblasts Electroporated with an α -sm Actin-specific mAb Exhibit Increased Motility

If the expression of α -actin filaments were indeed causative rather than correlative for migration potential, inhibition of incorporation of α -actin into filaments should stimulate migration. To address this question, an electroporation procedure (25) was optimized in our system. A field strength of 625 V/cm with a capacitance of 960 μ F has been shown to give the highest loading (1.25×10^{-15} M/cell of up to 150-kD molecules) at the lowest expense of viability (80%) of human fibroblasts (25).

Two antibodies specific to β -actin, A5441 and α -sm actin, 1A4, respectively, were used (Fig. 3 A). Whereas β -actin was present in both stress fibers, including retraction fibers and lamellipodia, α -sm actin was expressed predominantly in stress fibers, and in particular in retraction fibers (Fig. 3 B). Upon electroporation of A5441, some stress fibers were decorated as if they remained intact, and some staining was seen in the cytoplasm (Fig. 3 C). In contrast, electroporation of 1A4 resulted in depletion of α -sm actin from filaments in cells with thin filaments only (not shown). In cells with initially more prominent α -sm actin filaments, some filaments remained almost intact, while yet displacing the majority of α -sm actin to the cytoplasm in the form of small aggregates (Fig. 3 Cb) (9, 59). These observations may be analogous to studies in smooth muscle cells showing immediate disappearance of α -sm actin in thin actin filaments only (59). Accordingly, the retraction fibers of myodifferentiated fibroblasts are less easily depleted (compare Fig.

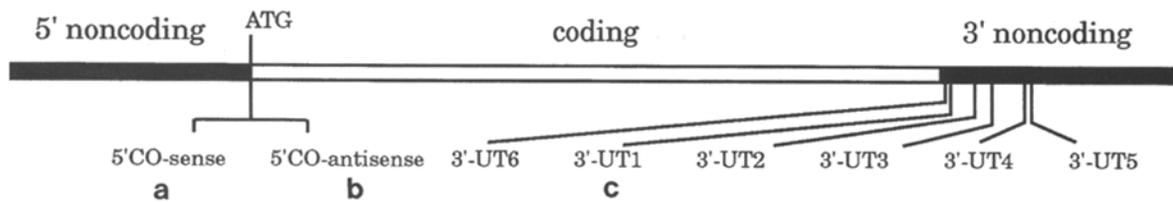
3, B and C). Thus, in contrast to what has been described *in vitro*, 1A4 *in vivo* counteracts incorporation of α -sm actin into filaments, suggesting that specific actin-binding proteins may exist (9). That nonmuscle actin filaments in fact persisted in spite of such α -sm actin depletion was revealed by FITC-phalloidin and β -actin staining (not shown). We assayed the electroporated cells for up to 3 d. During this period, the introduced antibodies were not degraded intracellularly as revealed by preserved antigenicity in the cytoplasm (not shown). Moreover, it has been shown by others that within this period, electroporated 1A4 almost completely blocks the contractile potential of α -sm actin-positive fibroblasts (2).

In a time course study, significant increase in migration was obtained at both 24 and 48 h after poration of 1A4 vs an unrelated antibody, Z259 (Fig. 3 D). Similar results were obtained by time lapse video microscopy (Fig. 3 E). Accordingly, in cells electroporated with 1A4, thin filaments were depleted of α -sm actin during this period (not shown). No significant difference was found in migration of cells electroporated with β -actin mAb vs Z259. This may reflect that anti- β -actin tags both stress fibers and lamellipodia such that the balance between the two compartments is maintained. Alternatively, the antibody is absorbed in the large globular pool of β -actin; in comparison, α -actin is preferentially confined to filaments (44). In a control experiment, electroporation of 1A4 failed to increase motility of fibroblasts without α -sm actin (not shown).

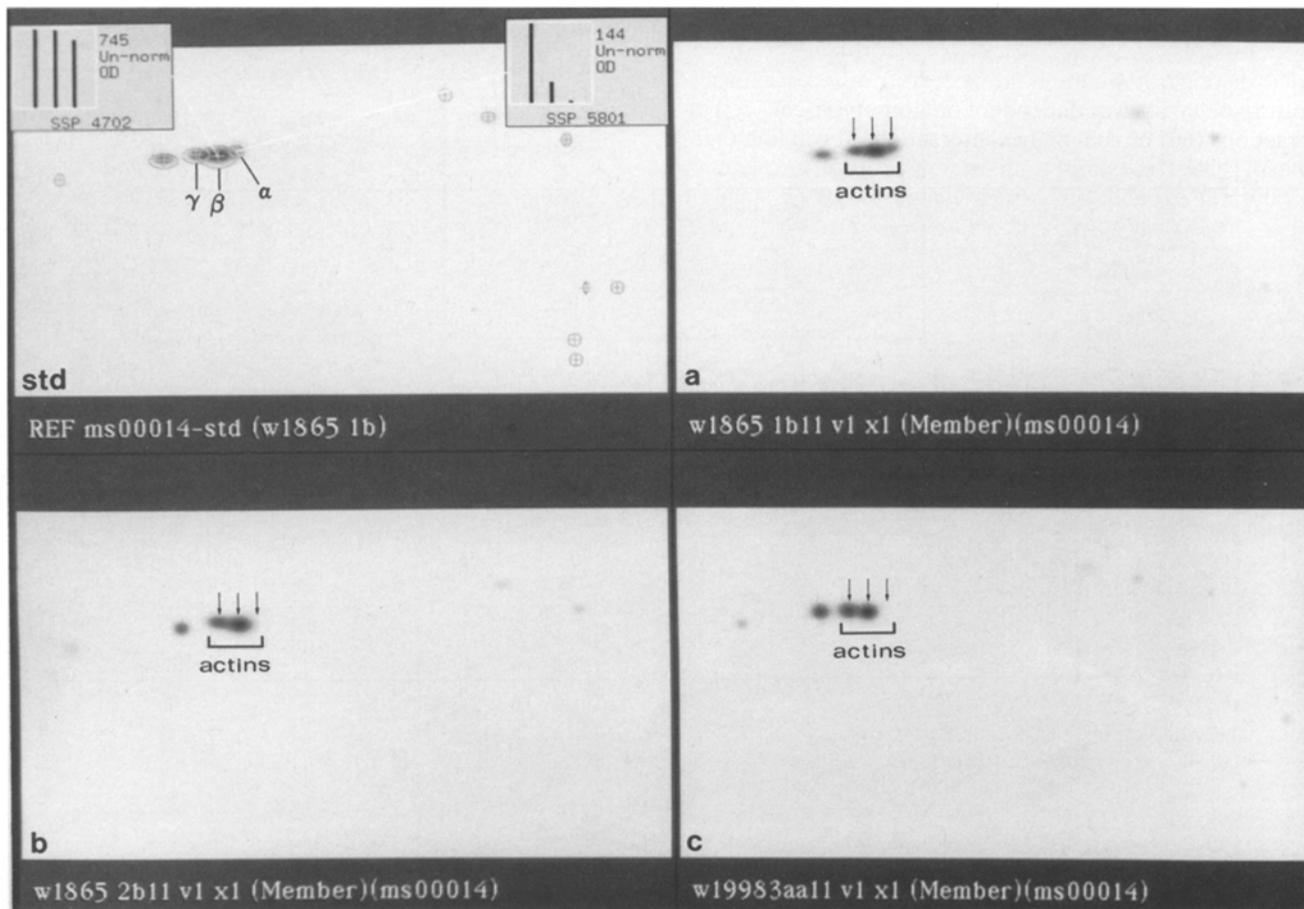
The Use of α -sm Actin Antisense ODNs

Seven 20–25-mer phosphorothioate modified antisense ODNs were designed. A number of previous studies have suggested that antisense ODNs against sequences adjacent to the ATG translation initiation codons are most effective in inhibiting translation (39, 67). Others have found the most significant reduction with ODNs that targeted specific sequences in the 3' UT region of the mRNA (10, 65). For actin genes, the 3'UT region is isoform specific and is believed to play a central role in compartmentalization of actins (33, 47). We designed one of our ODNs to span the ATG translation initiation codon (codons –2–6) unique to the human smooth muscle α -actin gene (Fig. 4 A; 4). The others encompassed six different α -sm actin-specific positions of the 3'-downstream segment of the human α -sm actin-encoding gene (Fig. 4 A; 32).

The seven ODNs were initially screened for their ability to inhibit α -sm actin synthesis as revealed by [35 S]methionine incorporation and fluorography of two-dimensional gels. A variable inhibition was recorded with the four of the antisense 3'UTs (3'UT2, 3, 4, 6), and one of them, the 3'UT5 antisense, elicited a general, nonspecific inhibitory effect (not shown). Only two of the antisense ODNs, 5'CO-antisense and 3'UT1, elicited a significant inhibition of α -sm actin synthesis. Administration of the 5'CO-sense ODN had no effect on α -sm actin synthesis. A similar result was obtained with a noncomplementary 5'CO (not shown). Therefore, a match set including a computerized standard of 5'CO sense, 5'CO antisense, and 3'UT1 antisense was generated in the PDQUEST software (Fig. 4 B). Quantification of autodetected spots showed an OD of



A

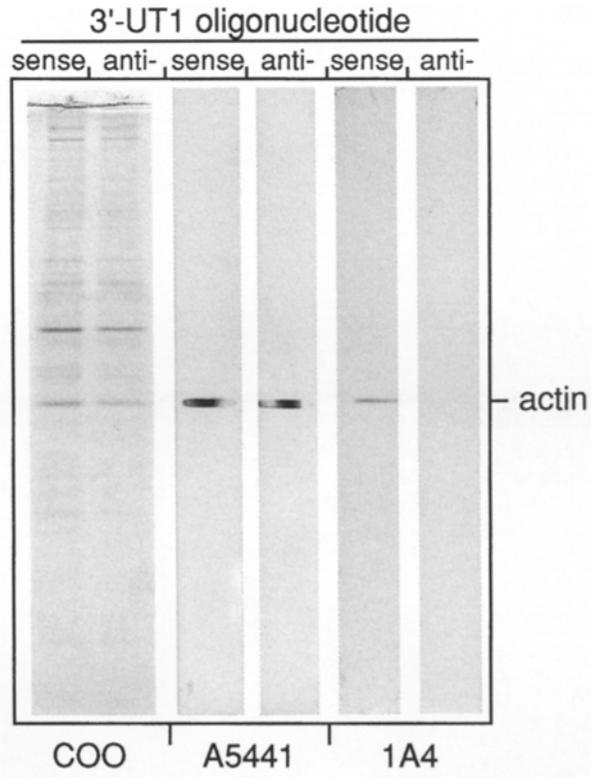


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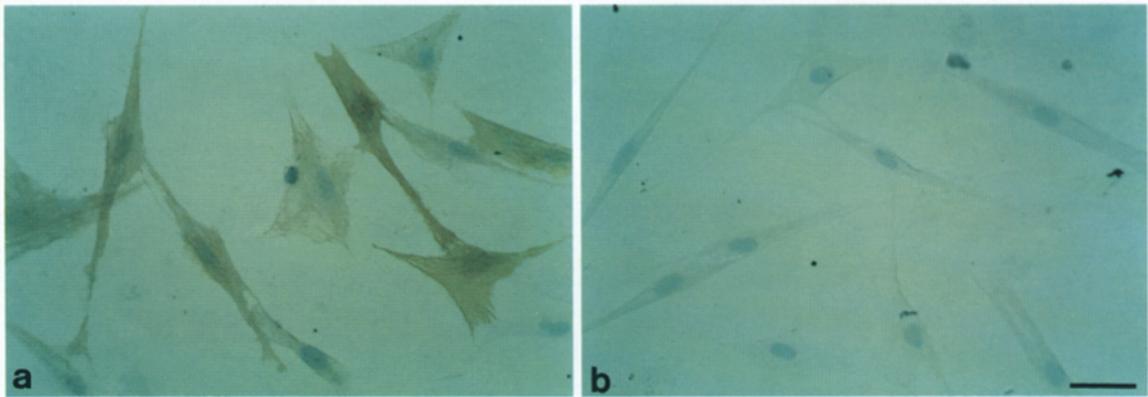
Figure 4. Antisense repression of α -sm actin protein synthesis. (A) Schematic representation of the cDNA regions complementary to mRNA targeted by ODNs. (B) Screen image of match set and computerized standard of two-dimensional gel fluorographies of ODN-electroporated, [^{35}S]methionine labeled α -sm actin-expressing fibroblasts. The computerized standard (*std*) shows spot detection by cross hairs and circles. The γ -, β - and α -actin spots are indicated, and histograms of OD of actin spot in *a*, *b*, and *c* (left, middle, and right bar, respectively) are projected in the upper left corner for β -actin and in the upper right corner for α -sm actin. (a) Note the strong α -sm actin synthesis in cells electroporated with the 5'CO sense ODN. (b) The 5'CO antisense elicited an incomplete repression of α -sm actin synthesis. (c) The 3'UT1 antisense ODN directed against a downstream segment of the 3'-untranslated α -sm actin mRNA—a segment with extensive interspecies homology—clearly elicited a complete repression of all α -sm actin synthesis without any interference with other translational events.

~ 745 arbitrary units (a.u.) in the β -actin spots. Thus, β -actin synthesis was not decreased by antisense treatment to any significant degree (Fig. 4 B, *std*, upper left). The OD of the α -sm actin spot of the sense-treated cells was 144 a.u., corresponding to $\sim 20\%$ of the β -actin spot, and in con-

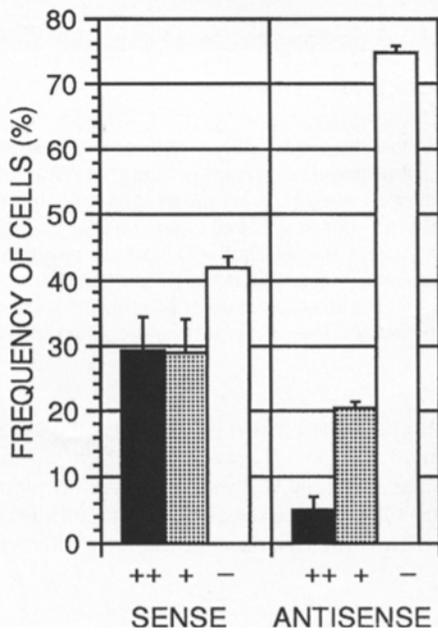
trast to β -actin, this level was significantly reduced by antisense treatment (Fig. 4 B, *std*, upper right). Only the 3'UT1 antisense was able to completely inhibit α -sm actin synthesis. This ODN tags a sequence in the middle of a 31-bp region, which is highly conserved among different spe-



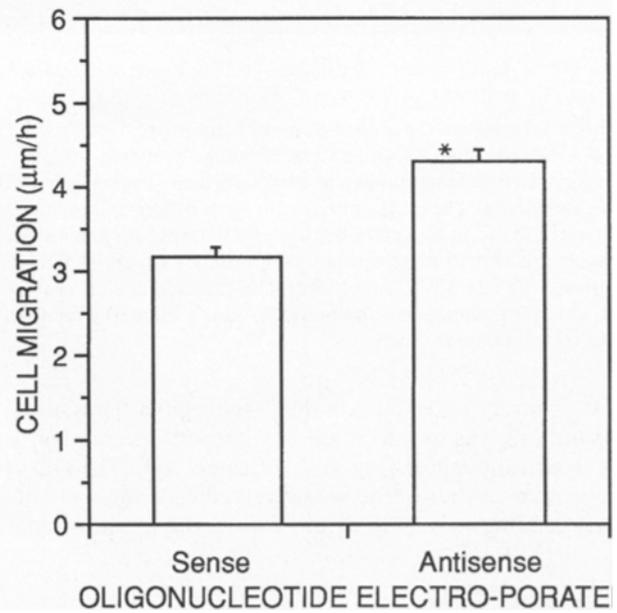
A



B



C



D

cies. The sequence homology has led to speculations that this particular region has a regulatory function for tissue-specific expression during development (32). The 3'UT1 was selected for further analysis. First, we tested whether antisense blockage by 3'UT1 was entirely specific for α -sm actin compared with the corresponding sense ODN. By two-dimensional gel electrophoresis of synthesized proteins, only one single spot, that of α -sm actin, was affected to any significant degree (not shown). A similar entirely specific, although partial, inhibition was recorded for the 5'CO antisense/sense match (not shown). A dose-response analysis revealed that the optimal blockage of α -sm actin was obtained at 10 μ M ODN. A higher concentration (20 μ M) was not toxic, but did not add any further to the effect (not shown). A time course study demonstrated that introduction of the α -sm actin-suppressing antisense resulted in an immediate total inhibition of α -sm actin synthesis that lasted for >48 h compared with the sense ODN. The effect of the antisense ODN was reversible since compensatory synthesis of α -sm actin was resumed between 48 and 72 h after administration. The time course of synthesis in sense-treated cells corresponded to that of cells that were neither exposed to ODN nor electroporated (not shown).

Depletion of α -sm Actin by Administration of Antisense 3'UT1

We next used the 3'UT1 antisense/sense ODNs to generate paired samples of α -sm actin-negative and -positive fibroblasts. To circumvent the problem with residual protein due to long turnover rates as often seen with cytoskeletal proteins, and in particular with α -sm actin (3, 42, 67), fresh fibroblasts, which are initially devoid of α -sm actin expression, were used (49, 52). After exposure to ODNs, the cells were further stimulated for 72 h by TGF- β 1 (49) in the presence of ODN. The fibroblasts treated with the sense ODN had readily appreciable levels of α -sm actin induced, whereas those treated with the antisense remained essentially negative as revealed by both immunoblotting and immunocytochemistry (Fig. 5). In the presence of sense ODN, up to 60% of the cells had α -sm actin induced, whereas in the presence of antisense ODN, <25% had α -sm actin induced (Fig. 5 C). Densitometric analysis of immunoblottings revealed equal reflection density (RD) of β -actin bands with sense and antisense, i.e., 1.57 versus 1.62 RD. In comparison, the density of the α -sm actin bands were 0.35 RD (corresponding to \sim 22% of the β -actin level) with sense vs 0 RD with antisense. Similar results were obtained with fibroblasts stimulated for 24 h with 20% FCS after electroporation.

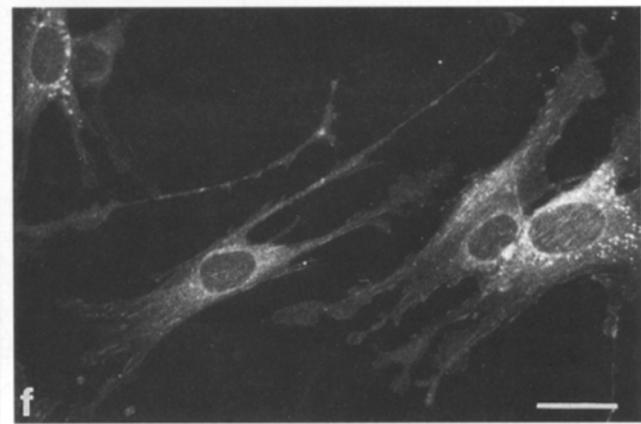
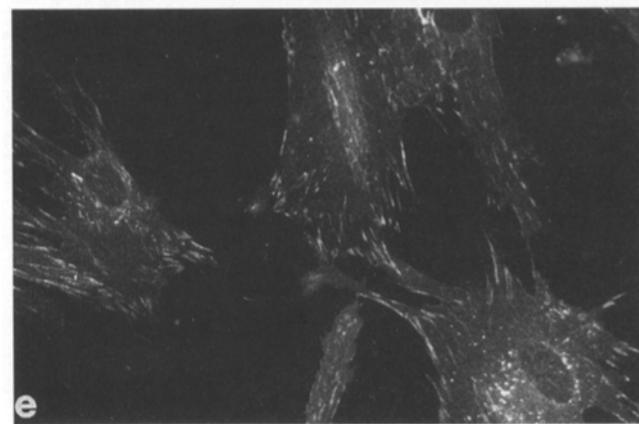
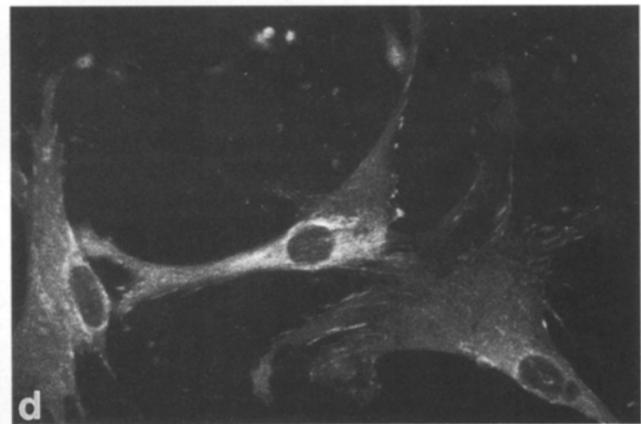
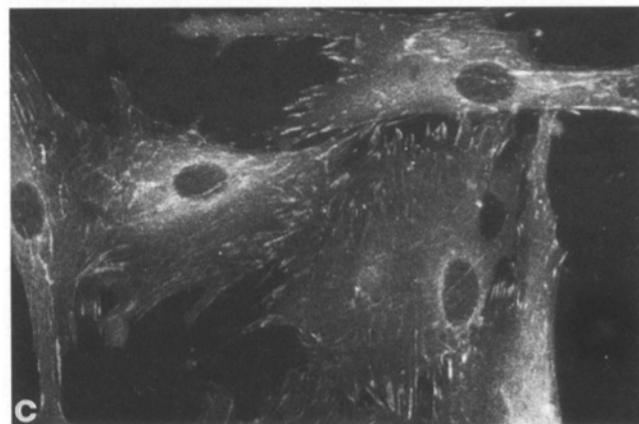
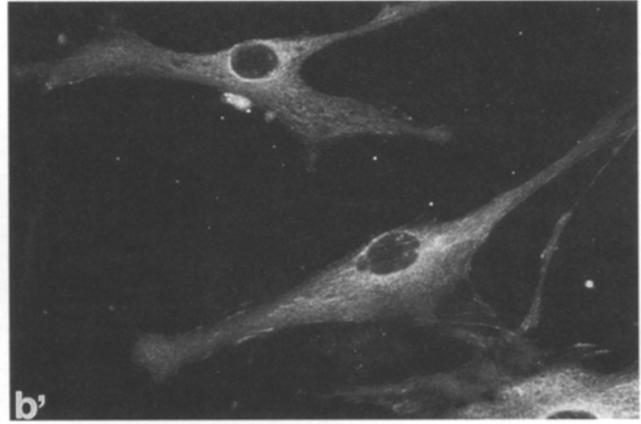
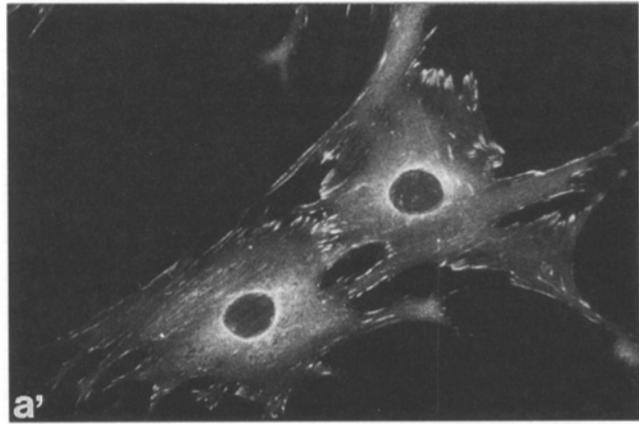
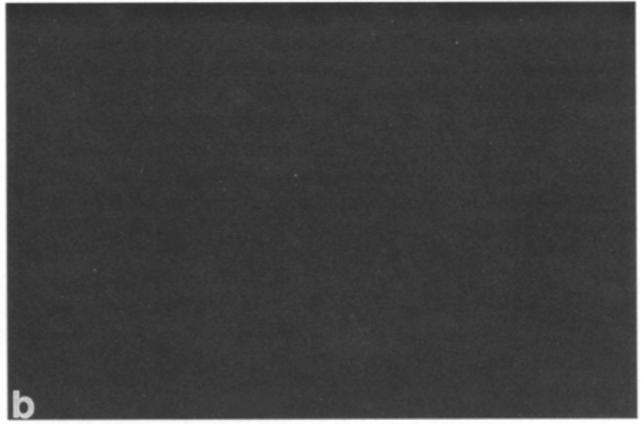
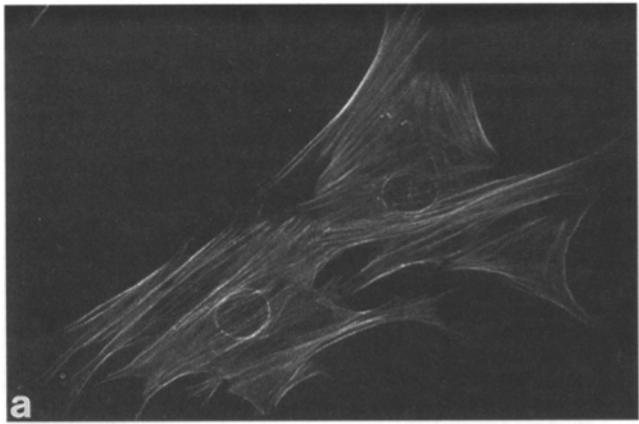
3'UT1 Antisense Lifts the Migratory Restraint of α -sm Actin-expressing Fibroblasts

To finally investigate whether inhibition of α -sm actin synthesis by antisense ODNs had any effect on fibroblast motility, migration was measured by time lapse video microscopy in the 24–35-h period after exposure to ODNs. In comparison with cells exposed to 3'UT1 sense, 3'UT1 antisense induced a morphological change corresponding to a more motile, i.e., more elongated, phenotype. This difference was somewhat more distinct than in cells electroporated with 1A4 and Z259, respectively, but morphology in itself may not consistently predict motile behavior (27, 56). However, migration track analysis revealed that 3'UT1 antisense significantly enhanced fibroblast motility by 27% (Fig. 5 D). Similar results were obtained with the partially suppressive 5'CO antisense ODN; motility was significantly enhanced by 12.5% with 5'CO antisense relative to 5'CO sense (data not shown). Thus, these data support a stoichiometric correlation between the potential of antisense ODNs to suppress α -sm actin synthesis and their ability to promote fibroblast motility. Migration of fibroblasts devoid of α -sm actin was not influenced by antisense ODNs (data not shown). It is noteworthy that knowledge of the mechanism of action of 3'UT ODNs in general is limited. One possibility is that the mRNA is destabilized either by RNase H-dependent mechanisms or by modulation of the natural processes that help to stabilize the mRNA, as has been shown for an ODN binding to the 3'UT region of ICAM-1 (10). Alternatively, 3'UT ODNs may delocalize the complementary mRNA, which in the case of β -actin has been shown to lead to changes in actin stress fiber organization (34). However, in contrast to the 3'UT ODN used in the present study, β -actin mRNA or protein levels were not affected (34). Whether delocalization of α -sm actin mRNA in itself leads to inhibition of synthesis remains to be established.

The State of α -sm Actin Assembly in Stress Fibers Regulates Focal Contact Formation

We next sought a mechanistic explanation for how, at the molecular level, filamentous α -sm actin may retard motility. With our previous findings in mind that expression of α -sm actin correlated with focal contact formation (Fig. 2), we analyzed whether antisense ODN depletion of α -sm actin influenced the organization of focal contacts. Similar to the considerations regarding residual protein due to long turnover rates as mentioned above, in these experiments, fibroblasts initially devoid of α -sm actin expression were used. Stress fibers were present in both 3'UT1 anti-

Figure 5. 3'UT1 antisense ODN significantly reduces the level of α -sm actin protein in fibroblasts. Fibroblasts were stimulated for 48 h with 20% FCS before electroporation of 10 μ M ODN, followed by another 72 h in serum-free medium with 100 pg/ml TGF- β 1 and ODN. (A) Immunoblotting of lysed fibroblasts electroporated with 3'UT1 sense or antisense ODNs and stained for β -actin (A5441) and α -sm actin (1A4). Whereas the β -actin staining stays essentially unaffected, the α -sm actin is significantly reduced. COO, Coomassie blue staining to indicate loading. (B) Immunoperoxidase staining of fibroblasts electroporated with 3'UT1 sense (a) and antisense (b) ODNs. Note the difference in staining intensity. (C) Histogram showing the suppressive effect of 3'UT1 antisense ODN on α -sm actin expression. Fibroblasts were stained by immunoperoxidase cytochemistry with mAb 1A4, and 3 \times 100 cells were counted in randomly selected fields and distributed into three categories: strong staining (++, black bar), intermediate staining (+, shaded bar), and no staining (-, open bar) (mean \pm SD of percentages; n = 3 experiments). (D) Mean migration in microns per hour based on video time lapse recording of three different experiments, each including \sim 75 initially α -sm actin-positive cells electroporated with either 3'UT1 sense or antisense. The difference is shown to be significant (*P < 0.05) using an unpaired Student's *t* test with two tails.



sense- and sense-exposed fibroblasts as revealed by double-staining for α - and β -actin (not shown). However, only 3'UT1 sense-exposed fibroblasts expressed α -sm actin in prominent stress fibers (Fig. 6 *a*). In spite of the presence of vinculin, talin, and β 1-integrin in both conditions at apparently equal amounts, they failed to organize in focal contacts in fibroblasts exposed to 3'UT1 antisense (Fig. 6).

Discussion

Resting, nonactivated fibroblasts of all tissues only express the two nonmuscle actin isoforms, β - and γ -actin. Any activation of fibroblasts under reactive conditions leads to induction of α -sm actin (12, 14, 15, 49, 51, 52; for review see reference 50). Importantly, however, in myodifferentiated fibroblasts, α -sm actin comprises only \sim 14% of the total actin content compared with up to 60–70% in vascular smooth muscle cells (2, 41, 61). The most obvious and anticipated function of α -sm actin, apart from that of contraction (2), has been that it has something to do with increased cellular migration (72). Thus, α -sm actin expression has repeatedly been spatially and temporally correlated with cells participating in tissue remodeling such as embryonic mesenchymal cell migration, wound healing, and planaria regeneration (12, 14, 17, 43). Since actin in general is known to be a motor protein in cell motility, it is logical to assume that increased expression of any of its isoforms would likely lead to increased motile behavior. Here, we demonstrate the opposite: that expression of one of the isoforms, that is, filamentous α -sm actin in fibroblasts, leads to decreased motility. We show that α -sm actin-negative cell strains migrate at a higher speed than corresponding α -sm actin-positive strains. Moreover, depletion of α -sm actin by specific antibody electroporation or antisense ODN repression leads to a significantly enhanced motility. The fact that focal contacts form and organize in a coordinate manner relative to α -sm actin expression in prominent stress fibers offers a logical explanation for the observed differences in motile behavior.

Nevertheless, there are a number of studies that appear to lend credence to these results: It has been shown that whereas β -actin predominates in the highly motile lamellipodia, α -sm actin is restricted to the less motile stress fibers (13, 26). This has been interpreted in favor of the idea of a functional sorting of actin isoforms and is in direct support of our data. This becomes even more plausible if one considers the widely held view that fibroblasts are among the least motile cells, precisely because of their elaborate stress fiber phenotype (62). The mean speed of fibroblast movement has been determined to be 4–7 μ m/h, not very different from our data, but fibroblasts are generally considered as almost immobile compared with most other cell types moving at >10 μ m/h (16). A drastically different understanding of this issue can be obtained when

the classical data on fibroblast motility are viewed against more recent data concerning the phenotype of fibroblasts used in such studies. In retrospect, it is reasonable to assume that all fibroblasts previously used in traditional culture conditions have expressed a more or less elaborate α -sm actin phenotype, and as such, in reality have been myofibroblasts. In other words, all fibroblast cultures tested so far (with the exception of a few strains, e.g., F359, F498, and F514 used here) express α -sm actin in the presence of serum (15, 52, 57, 63). Therefore, the well established association between stress fiber formation and immotile behavior may very well be due to incorporation of α -sm actin.

Once appreciated, this hypothesis fits nicely into a number of other observations peripheral to α -sm actin but related to the central issue of motility and stress fibers. Originally, compelling evidence was provided for the notion that whereas stationary cells expressed most of the actin in stress fibers, rapidly translocating cells showed only diffuse actin staining (27). More recently, it has been shown that transfection of a variant β -actin isoform with preference for stress fibers over motile areas of cytoplasm into highly metastatic melanoma cells leads to a reduction in both metastatic and motile behavior (58). Another example is rat embryo fibroblasts (REF 52-2), which show very prominent stress fibers and only little motility (23). Also, it has been shown that sarcoma cells may be divided into at least three phenotypes based on the organization of the actin cytoskeleton: those showing mostly cortical actin, those showing a network of short actin fibers, and finally those with well-developed stress fibers. The latter turned out to be the least motile (1 μ m/h) and the least metastatic (46). A similar array of data has been generated with primary cultures of human fibroblasts in which stress fiber formation was repressed by addition of newborn rat heart-conditioned medium (18). Among the cells with prominent stress fibers, \sim 40% migrated, whereas among the stress fiber-depleted cells, this frequency increased to \sim 60% (18).

If indeed a reduction in α -sm actin leads to retardation of motility, then it could be argued that just any reduction in the pool of filamentous actin would increase the rate of motility. This argument is particularly pertinent because an inverse correlation between cell migration speed in *Dictyostelium* and levels of filamentous actin has been demonstrated very recently by Hug et al. (30). In experiments to be reported elsewhere, we have attempted to narrow the cause-effect relationship between F-actin expression and motility in human fibroblasts by administering a required amount of the nonmuscle actin-severing toxin C2-toxin (1) to our migrating cells. Although a more thorough study including a wider range of toxin is desirable, our data (not shown) pointed to the fact that a reduction in the levels of nonmuscle β -/ γ -actin did not result in

Figure 6. Fibroblasts exhibit reciprocal relationship between α -sm actin expression and focal contact formation. Double-labeling immunofluorescence cytochemistry of fibroblasts stained for α -sm actin (*a* and *b*), vinculin (*a'* and *b'*), talin (*c* and *d*) and β 1-integrin (*e* and *f*), the latter two shown without the corresponding α -sm actin stainings. Fibroblasts were electroporated with either 3'UT1 sense (*a*, *a'*, *c*, and *e*) or antisense (*b*, *b'*, *d*, and *f*) ODNs. The state of α -sm actin assembly influences focal contact organization; thus inhibition of α -sm actin by 3'UT1 antisense leads to reduction in the number and size of focal contacts as assessed 24 h after electroporation. Magnification: 440. Bar, 25 μ m.

an increase in cellular motility. This was true for a reduction both corresponding to the level of reduction in α -sm actin and also for even a larger reduction in nonmuscle actin. This finding is compatible with the fact that α -actin has a preference for F-actin compared with the nonmuscle isoforms (44 and Fig. 3 B), and that α -sm actin is more competent for polymerization than the other isoforms (9). Therefore, any reduction in α -sm actin would decrease F-actin relatively more than the same reduction in β -/ γ -actin. As such, a reduction in α -sm actin in fibroblasts may lead to the same end result observed in *Dictyostelium*, i.e., an increase in cell migration by overexpression of capping protein (30).

How the organization of the cytoskeleton is translated into motile behavior remains an open question. The prevailing concept operates with three general characteristics of the cell phenotype. First, the actin cytoskeleton generates the intracellular mechanical stresses. Second, cell-substratum traction created by dynamic adhesion processes transforms these stresses into a displacement of the cell body. Third, morphological polarization channels this force unidirectionally as required for cell body translocation (16). For this entire concept to be operational, it has to rely on a biphasic relation between cell adhesion and motility. In other words, migration is only optimal at a certain level of attachment (1.5 – $3.8 \mu\text{dynes}/\mu\text{m}^2$) beyond or below which motility decreases (16). Accordingly, both overexpression and repression of vinculin leads to the same end result, i.e., decreased motility (20, 21, 70). We show here that α -sm actin expression in less motile fibroblasts correlates strongly with the deposition of vinculin as well as talin and $\beta 1$ -integrin at prominent focal contacts. Interestingly, it has been shown that there is a feedback loop between the state of actin assembly and vinculin synthesis, so that when there is more assembled actin, there is higher vinculin synthesis and larger focal contacts (5). Indeed, there seems to be a regular inverse relationship between the size of stress fibers and the area occupied by focal contacts ($0.4 \mu\text{m}^2$ – $1.3 \mu\text{m}^2$) on the one hand and motile behavior on the other (46).

The fact that focal contacts are downregulated coordinately with α -sm actin in our antisense ODN repression experiments is not entirely incompatible with previous experiments with injected proteins capping the distal ends of actin (31). This is further supported by another study comparing normal human fibroblasts with those from individuals with inherited colon adenomatosis. The fibroblasts from adenomatosis individuals failed to develop well-defined vinculin containing focal contacts. However, this was not due to a difference in the cellular content of vinculin, but rather to a perturbed stress fiber formation in the adenomatosis fibroblasts (28).

Although it may be reasonable to assume from these considerations that many previous data on stress fibers in fibroblasts may in fact be taken to represent data on α -sm actin, only a few studies have focused directly on this isoform. Time course studies *in vivo* clearly reveal that α -sm actin expression and focal contact formation occur concomitantly around days 4–7 during wound healing (29). In terms of motility, this temporal expression coincides with the time when fibroblasts reach their prefinal destination (after the main cell translocation) in the newly formed

granulation tissue completely covered with regenerating epithelium. This corresponds to the time when cells are needed so that contraction can occur. α -sm actin may play a similar role in the stromal reaction in cancer, such as breast carcinoma (for review see reference 50).

Finally, if our argument that α -sm actin serves as a brake in activated fibroblasts is valid for all tissues, this would imply that activated fibroblasts that for some reason lacked α -sm actin would behave in an uncontrolled manner. Although circumstantial, it is worth noting that the most prominent single molecular event among malignantly transformed rat and mouse fibroblasts is a total shutdown of α -sm actin (35, 40). The transformed cells with residual α -sm actin expression apparently do not metastasize. In the light of current knowledge on the link between tumor cell motility and metastasis (64), and the results presented here, it is tempting to speculate that the permanent lack of α -sm actin in transformed cells will in fact result in constitutive enhanced motility.

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