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FAST TRACK

Autoregulation of Actin Synthesis Requires the 3′-UTR of Actin mRNA and Protects Cells from Actin Overproduction

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Abstract Monomeric (G) actin was shown to be involved in inhibiting its own synthesis by an autoregulatory mechanism that includes enhanced degradation of the actin mRNA [Bershadsky et al., 1995; Lyubimova et al., 1997]. We show that the 3′-untranslated region (3′-UTR) of β-actin mRNA, but not its 5′-untranslated region, is important for this regulation. The level of full-length β-actin mRNA in cells was reduced when actin filaments were depolymerized by treatment with latrunculin A and elevated when actin polymerization was induced by jasplakinolide. By contrast, the level of actin mRNA lacking the 3′-UTR remained unchanged when these drugs modulated the dynamics of actin assembly in the cell. Moreover, the transfection of cells with a construct encoding the autoregulation-deficient form of β-actin mRNA led to very high levels of actin expression compared with transfection with the control actin construct and was accompanied by characteristic changes in cell morphology and the structure of the actin cytoskeleton. These results suggest that the autoregulatory mechanism working via the 3′-UTR of actin mRNA is involved in controlling the maintenance of a defined pool of actin monomers that could be necessary for the proper organization of the microfilament system and the cytoskeleton-mediated signaling. J. Cell. Biochem. 76:1–12, 1999.

Key words: actin autoregulation; mRNA degradation; 3′-UTR; jasplakinolide; latrunculin A; actin cytoskeleton

Actin is the most abundant protein in most eukaryotic cells, serving as the building block of the ubiquitous cytoskeletal fiber system known as the microfilaments. The microfilaments are involved in a variety of important cellular functions, including maintenance of cell shape and intracellular organelle positioning in the cell, transport of molecules and vesicles, cytokinesis, cell adhesion and motility, and others [Bershadsky and Vasiliev, 1988; Bray, 1992]. The level of actin in the cell was therefore expected to be finely balanced to carry out these multiple functions in an optimal manner. Indeed, the rate of actin production was found to be regulated at multiple levels. Transcriptional regulation of the actin gene was demonstrated in cells stimulated with growth factors [Elder et al., 1984, 1988; Greenberg et al., 1985, 1986; Ryseck et al., 1989] and in response to cell adhesion to the substrate [Farmer et al., 1983; Dike et al., 1988]. In addition, post-transcriptional mechanisms controlling actin expression were also described. These include the observation that the stability of actin mRNA can be subject to an autoregulatory mechanism that is linked to the level of monomeric (G) actin in the cell [Bershadsky et al., 1995; Reuner et al., 1995b]. Recently, the level of actin was shown to be regulated post-translationally by ubiquitin-dependent degradation [Solomon and Goldberg, 1996; Bercovich et al., 1997].

The mechanism controlling actin mRNA stability by autoregulation is of particular interest, since this regulation is closely related to a major property of actin, that is, its ability to exist in two forms: a polymeric, or filamentous form (F-actin) and a monomeric, or globular form (G-actin). Most of the actin-related functions in the cell depend on the assembly of actin filament arrays from G-actin, in a time- and space-dependent process. The level of monomeric actin in the cytoplasm, however, signifi-

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cantly exceeds the critical concentration for actin polymerization determined under physiological salt conditions in vitro [Theriot, 1994]. This very high level of nonpolymerized actin monomers in the cell is achieved, at least in part, by the binding of actin monomers to thymosin β4 and profilin. A balance between these monomeric actin-sequestering proteins, and most probably other factors, controls the dynamics of actin assembly in cells [Carlier and Pantaloni, 1994].

In previous studies, we demonstrated that the monomeric, but not filamentous, actin is part of the negative regulation that controls the level of actin mRNA by decreasing its stability [Bershadsky et al., 1995; Lyubimova et al., 1997]. Treatment of cells with latrunculin A, which causes depolymerization of actin filaments and increases the level of G-actin [Spector et al., 1983; Coué et al., 1987], leads to a reduction in actin mRNA level. By contrast, treatment with swinholide A [Carmeli and Kashman, 1985], which also depolymerizes actin filaments, but sequesters actin in a dimeric form [Bubb et al., 1995], results in increased levels of actin mRNA. The actin assembly-dependent mechanism that regulates actin mRNA therefore responds to the level of monomeric actin and not to that of the filamentous form of actin [Lyubimova et al., 1997].

The molecular mechanism(s) underlying this autoregulation are still largely unknown. It is also unclear whether the mechanism that brings about the decrease in actin mRNA level after the increase in the concentration of G-actin is the same as the one inducing the increases in actin mRNA content after G-actin levels are reduced. Moreover, since the experiments in which actin dynamics were modulated are based on the treatment of cells with various inhibitors, it is unclear whether this autoregulatory feedback can function in cells that were not treated with drugs.

In the present study, we employed various deletion mutant constructs of actin mRNA in transfection studies and showed that the monomeric actin-mediated regulation of actin expression depends on sequences localized in the 3′-untranslated region (3′-UTR) of actin mRNA. Deletion of the 3′-UTR renders actin mRNA insensitive both to the increase and to the decrease in the level of G-actin in cells. Moreover, transfection of cells with a construct lacking the 3′-UTR led to a dramatic increase in actin expression, as compared with transfection with 3′-UTR-containing actin, and was accompanied by severe aberrations in cell morphology and the structure of the actin cytoskeleton.

MATERIALS AND METHODS

Cell Culture and Drug Treatment

Balb/C-3T3 clone A31 and SV-80 cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% calf serum (Gibco, Grand Island, NY) at 37°C in a humidified atmosphere with 7.5% CO_2, in the presence or absence of latrunculin A (10 μM, a gift from Dr. I. Spector, SUNY, Stonybrook, NY), or jasplakinolide (200 nM, Molecular Probes, Eugene, OR).

Transient Expression of cDNA in Cultured Cells

cDNA constructs were transfected into SV-80 cells by the calcium phosphate method. At 30 h post-transfection, the cells were treated with various drugs for the indicated time periods. The expression of endogenous and transfected actin mRNA was examined by reverse transcription–polymerase chain reaction (RT-PCR) (see below).

Reverse Transcription Followed by PCR

cDNAs for full-length human β-actin (HF) and human β-actin lacking the 5′-UTR and 3′-UTR (Δ5′-UTR and Δ3′-UTR) were transiently expressed in SV-80 cells. At 24 h post-transfection, the cells were treated with latrunculin A for 12 h and total RNA extracted by the Trireagent method. Total RNA (500 ng) was used for reverse transcription (RT) by Super-Script II Reverse Transcriptase (Gibco) using sets of specific oligonucleotide primers designed for the mRNA of interest (see below). Equal volumes of each cDNA mixture were used for PCR using DynaZyme II DNA polymerase (Fynnzymes OY, Finland) with oligonucleotide primers. The concentration of cDNA and the number of PCR cycles were adjusted to obtain sufficient PCR products at the exponential stage of the amplification step. Equal volumes from each reaction were analyzed by electrophoresis on 1% agarose gels.

Cell Fractionation

Cells cultured on 35-mm dishes (5×10^5 cells/dish) were washed with PBS at room temperature and extracted with 0.5 ml of 50 mM

MES pH 6.8, 1 mM EGTA, 50 mM KCl, 1 mM $MgCl_2$, 1 mM PMSF, 0.1 mM aprotinin, 1 mM pepstatin, 0.1 mM leupeptin and 0.5% Triton X-100. After 1 min, the Triton X-100-soluble fraction was removed. The Triton X-100 insoluble fraction was scraped off the dishes into the same buffer. Both fractions were concentrated by ethanol precipitation and resuspended in 100 µl of Laemmli's sample buffer [Laemmli, 1970] and equal volumes (20 µl) of the lysates were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Radioactive Labeling of Cellular Proteins and Gel Electrophoresis

After treatment with the drugs, the cells were pulse-labeled with 200 µCi/ml of [^{35}S]-methionine for 30 min in methionine-free DMEM in the presence of the various drugs and the newly synthesized proteins analyzed by two-dimensional gel electrophoresis as described [Ben-Ze'ev, 1990].

Immunoblotting

Proteins were separated by SDS-PAGE and electrophoretically transferred from gels to nitrocellulose. Actin and tubulin were visualized on the blots with monoclonal anti actin (AC-15, Sigma Immunochemicals), or antitubulin (DM-1A, Sigma Immunochemicals) antibodies, followed by secondary horseradish peroxidase (HPO)-conjugated antibody (Amersham). The blots were developed using enhanced chemiluminescence (ECL).

RNA Isolation and Hybridization With cDNA

Total RNA was isolated by the Trireagent method and hybridized with [^{32}P]-dCTP-labeled cDNA as described [Ben-Ze'ev et al., 1990]. The following cDNA probes were used: mouse β-actin [Farmer et al., 1983], human β-actin coding region [Gunning et al., 1983], γ-actin 3′-UTR [Erba et al., 1986] and GAPDH [Dani, 1984].

Immunofluorescence

Cells cultured on glass coverslips were permeabilized and fixed for 2 min with a mixture of 0.5% Triton X-100 and 3% paraformaldehyde in PBS and further fixed for 20 min with 3% paraformaldehyde without detergent. Actin filaments were stained with tetramethyl rhodamine isothiocyanate (TRITC)- or fluorescein isothiocyanate (FITC)-labeled phalloidin (Sigma). Transfected actin tagged with the hemagglutinin (HA) epitope was visualized by indirect immunofluorescence using monoclonal anti-HA tag antibody from Babco (Richmond, CA) and TRITC-conjugated goat anti-mouse antibody from Jackson Laboratories (West Grove, PA). The cells were examined by epifluorescence using a Zeiss Axiophot microscope equipped with a ×100/1.3 Plan Neofluar lens and photographed using Kodak T-max p3200 film.

Construction of Plasmids

All plasmids were grown in *Escherichia coli* strain XL1-Blue. The full-length β-actin cDNA construct (FL) was cloned into the *Xho*I site of pcDNA3 (Invitrogen). The β-actin cDNA lacking the 3′-UTR (Δ3′-UTR) was constructed by PCR amplification using full-length β-actin cDNA as a template with primers containing *Hin*dIII (5′-CCAAGCTTCTGCAGATATCCATCACAC-3′) and *Xba*I (5′-ACG TCTAGACCTAGAAGCATTTGCGGTG-3′) restriction sites. The β-actin cDNA lacking the 5′-UTR was constructed by subcloning the β-actin coding region and the 3′-UTR into pcDNA3. The β-actin coding region was amplified using full-length β-actin cDNA as a template and a primers containing *Hin*dIII (5′-CCCAAGCTTATGGATGATGATATCGCC-3′) and *Bam*HI (5′-ACCTGGATCCCTAGAAGCATTTGCGGTGG-3′) restriction sites. The fragment corresponding to the 3′-UTR was obtained using primers containing *Bam*HI (5′-GCGGGATCCCGGACTATGAC TTAGTTG-3′) and *Xba*I (5′-ACGTCTAGAGCCT TCATACATCTCAAG-3′) restriction sites. The PCR products were subcloned into the *Hin*dIII/*Xba*I site of pcDNA3.

HA-tagged 3′-UTR-containing and 3′-UTR-deficient human β-actin cDNA constructs were prepared by subcloning the coding region and the 3′-UTR of β-actin (for the control construct), or the coding region alone (for the Δ3′-UTR construct), into the pCGN vector [Simcha et al., 1998] carrying an HA epitope.

RESULTS

Organization and Expression of Actin in Latrunculin A- and Jasplakinolide-Treated Cells

3T3 cells were treated with the F-actin-stabilizing drug jasplakinolide [Bubb et al., 1994] and compared with cells treated with

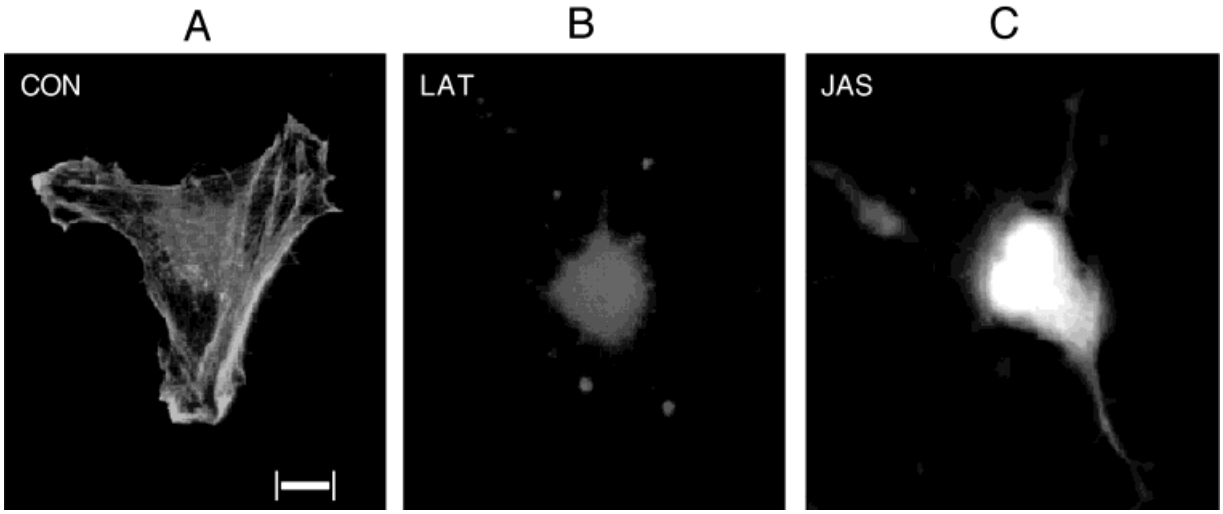

Fig. 1. Latrunculin A and jasplakinolide disrupt the organization of actin in SV-80 cells (**A**). Untreated cells (CON) and cells treated for 2 h with (**B**) 10 µM latrunculin A (LAT), or (**C**) 0.5 µM jasplakinolide (JAS) were fixed and stained with TRITC-phalloidin to visualize polymerized actin. Scale bar = 10 µm.

latrunculin A that causes F-actin disassembly into G-actin monomers [Spector et al., 1983; Coué et al., 1987; Bershadsky et al., 1995]. Latrunculin A treatment induced a retraction of the cell body, leaving behind long cellular processes, while stress fibers were not apparent in such cells (Fig. 1B, cf. control, Fig. 1A). Treatment with jasplakinolide resulted in the formation of cytoplasmic aggregates that were prominently stained with TRITC-phalloidin (Fig. 1C). Longer incubation periods with this drug led to cell rounding complete disruption of stress fibers and an increase in the number and size of the phalloidin-positive aggregates.

Analysis of the distribution of actin between a Triton X-100-soluble and an insoluble fraction showed that while in control cells about 50% of actin was in the Triton X-100-soluble fraction (Fig. 2, lanes 5 and 6), in latrunculin A-treated cells nearly all actin became Triton X-100 soluble (Fig. 2, lanes 1 and 2), while in jasplakinolide-treated cells, the majority of actin became Triton insoluble (Fig. 2, lanes 3 and 4). By contrast, treatment with these drugs had no effect on the distribution of tubulin between these two fractions, demonstrating their specificity toward the microfilament system.

To compare the effects of latrunculin A and jasplakinolide on actin synthesis, untreated cells and cells incubated with these drugs were labeled for 30 min with ^{35}S-methionine, and equal amounts of radioactive proteins were analyzed by two-dimensional gel electrophoresis.

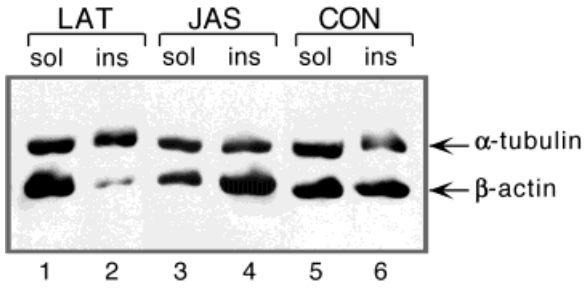

Fig. 2. Changes in the partitioning of actin between a Triton X-100-soluble and -insoluble subcellular fraction by treatment with jasplakinolide and latrunculin A. SV-80 cells were either left untreated (CON), or incubated for 12 h with jasplakinolide (JAS) and latrunculin A (LAT). Equal volumes from the Triton X-100-soluble (sol) (**lanes 1, 3, 5**) and -insoluble (ins) (**lanes 2, 4, 6**) fractions were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting with antibodies to β-actin and α-tubulin.

The results presented in Figure 3 show that while latrunculin A treatment induced a major decrease in newly synthesized β-and γ-actin (Fig. 3B, cf. Fig.3A,D), actin synthesis was significantly elevated in jasplakinolide-treated cells (Fig. 3C, cf. Fig. 3A,D). To determine whether these effects result from changes in actin RNA levels, Northern blots with RNA from control and drug-treated cells were hybridized with cDNA probes for β-actin. The results shown in Figure 4 demonstrate that while the level of β-actin RNA was reduced in latrunculin A-treated cells, in jasplakinolide-treated cells there was an increase in the level of β-actin

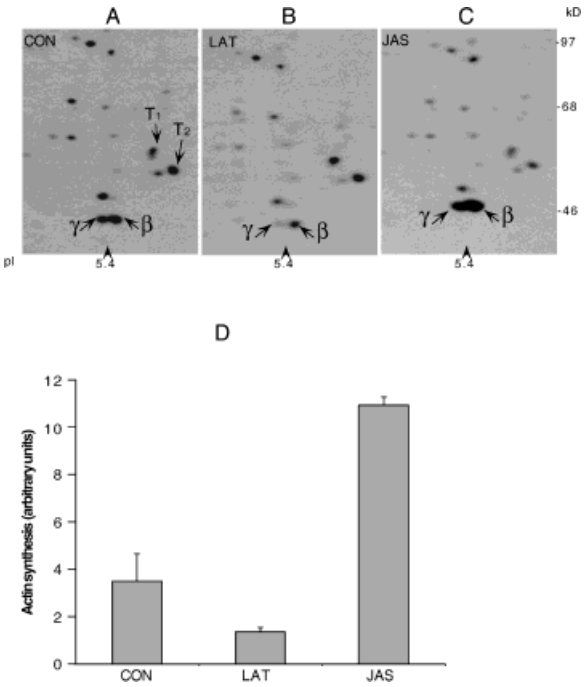

Fig. 3. Jasplakinolide and latrunculin A affect the synthesis of actin differentially. **A:** Control (CON) 3T3 cultures and cells incubated for 24 h with either (**B**) latrunculin A (LAT) or (**C**) jasplakinolide (JAS) were labeled for 30 min with ^{35}S-methionine; equal amounts of radioactive proteins were analyzed by 2-D gel electrophoresis. Actin synthesis was quantified using a GS-700 imaging densitometer. **D:** The level of actin was normalized to the levels of β-tubulin. The diagram represents means ±SD of determinations in two independent experiments. pI, isoelectric point; T_1 and T_2, α- and β-tubulin; β and γ, β- and γ-actin isoforms.

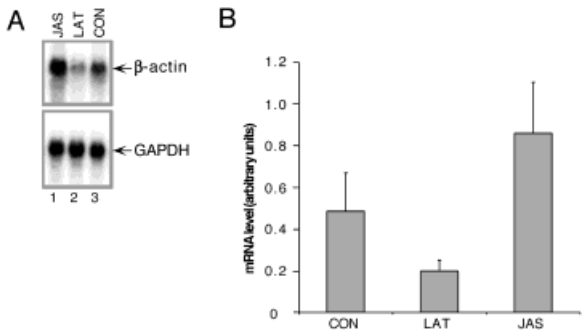

Fig. 4. Effects on actin RNA level in cells treated with latrunculin A and jasplakinolide. RNA was extracted from cells incubated with latrunculin A (LAT) (**A**, **lane 2**) or jasplakinolide (JAS) (**A**, **lane 1**) and from control (CON) 3T3 cells (**A**, **lane 3**). Northern blots containing equal amounts of RNA, per lane, were hybridized with a cDNA probe for β-actin, followed by rehybridization with a GAPDH cDNA. **B:** The levels of actin and GAPDH RNA were quantified by densitometry, and actin RNA content was normalized to the level of GAPDH RNA. Bars represent the mean ±SD from three independent determinations.

RNA (Fig. 4), implying that these changes in actin synthesis result from parallel changes in actin mRNA content.

Deletion of the 3′-UTR in β-Actin mRNA Disrupts the Regulation of Actin mRNA Turnover by Monomeric Actin

We have previously shown that the polymerization-dependent regulation of actin expression is confined to the cytoplasm and involves changes in actin mRNA stability [Bershadsky et al., 1995]. The stability of a variety of mRNA was shown to be dramatically altered by mutations introduced into either their 5′- or 3′-untranslated regions [Ross, 1995]. We have therefore examined whether the 5′- and/or the 3′-UTR of β-actin mRNA are involved in the monomeric actin-mediated actin mRNA degradation.

SV-80 cells were transiently transfected with either a full-length β-actin cDNA construct (FL), or with cDNA lacking either the 5′- or the 3′-UTR (Δ5′-UTR and Δ3′-UTR) of actin mRNA (Fig. 5A). At 30 h post-transfection, the cells were further incubated with latrunculin A or jasplakinolide for an additional 12 h, and total RNA was isolated from these cells. Equal amounts of RNA from each sample were analyzed by reverse transcription using specific oligonucleotide primers. The endogenous β-actin cDNA was extended from an oligonucleotide primer, P2, corresponding to its 3′-UTR (Fig. 5A). The transfected actin mRNA were extended from the oligonucleotide primer corresponding to the SP6 primer sequence in the expression plasmid (Fig. 5A). As control for transfection efficiency, part of the neomycin-resistance gene was extended from the neo-resistance reverse transcription primer. The concentration of cDNA and the number of PCR cycles were adjusted such that the amplification reaction was in the exponential phase of the PCR reaction.

The results shown in Figure 5B demonstrate that the levels of full-length actin and that of actin mRNA lacking the 5′-UTR were reduced in latrunculin A-treated cells as compared with control cells (Fig. 5B, lanes 3 and 5, cf. lanes 2 and 4). By contrast, the level of actin mRNA lacking the 3′-UTR remained unchanged after latrunculin A treatment (Fig. 5B, lanes 6 and 7). The level of the control Neo^r gene was similar in both drug-treated and untreated cells, suggesting that equal amounts of the transfected plasmid were expressed in the various

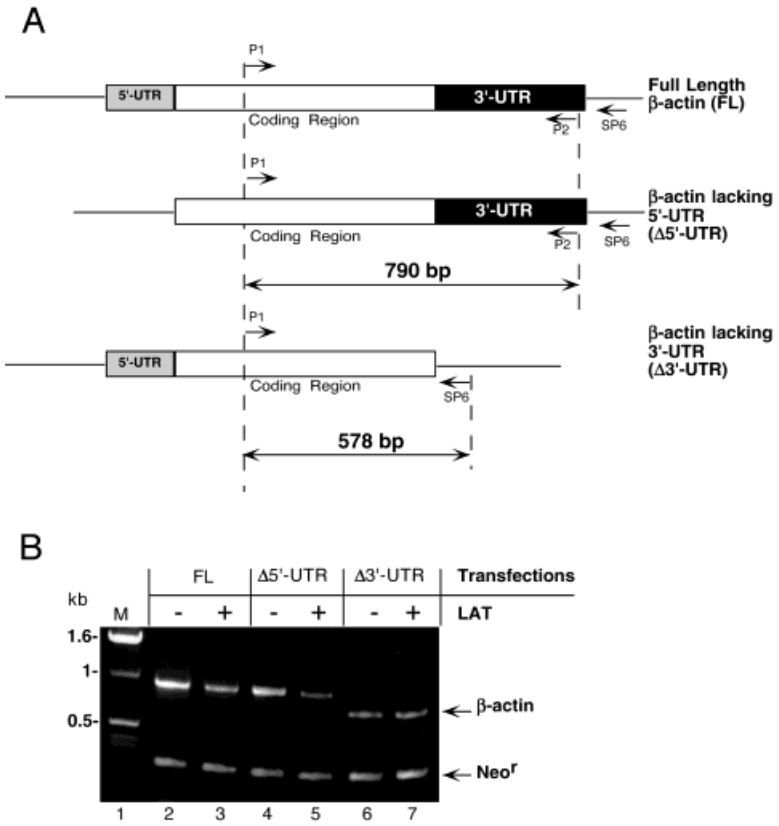

Fig. 5. Latrunculin A does not affect the level of actin mRNA that lacks the 3′-UTR. **A:** Diagram representing the transfected β-actin cDNA constructs. **B:** Levels of full-length β-actin mRNA (FL) and β-actin cDNAs lacking the 5′-UTR (Δ5′-UTR) and 3′-UTR (Δ3′-UTR) were determined by reverse transcription–polymerase chain reaction (RT-PCR) in cells transfected with these mRNAs; the cells were either left untreated for 30 h (**lanes 2, 4, 6**) or treated with latrunculin A (LAT) (**lanes 3, 5, 7**), 30 h after transfection, for 12 h. Neo^r, fragment from the neomycin resistance gene; M, molecular-weight markers.

samples. These results indicate that deletion of the 3′-UTR in β-actin mRNA rendered it resistant to the G-actin-inducible degradation, while deletion of its 5′-UTR did not affect the actin monomer-dependent regulation of actin mRNA level. Thus, the 3′-UTR of actin mRNA may contain a putative sequence that is involved in the regulation of actin mRNA degradation in response to an increase in the level of monomeric actin.

Previous studies have demonstrated that increased polymerization of actin that includes sequestration of monomeric actin can induce an elevation in actin mRNA content [Serpinskaya et al., 1990; Reuner et al., 1991, 1995a,b; Bershadsky et al., 1995; Lyubimova et al., 1997]. We have therefore examined whether the 3′-UTR of actin mRNA is also involved in this increase in actin mRNA level observed after the stabilization of F-actin by jasplakinolide. SV-80 cells were transiently transfected with either a full-length β-actin cDNA (FL), or with a cDNA lacking the 3′-UTR (Δ3′-UTR) and after 30 h incubated with either jasplakinolide or latrunculin A for an additional 12 h. The levels of the transfected FL- and Δ3′-UTR-encoded actin mRNA and that of endogenous β-actin RNA were determined in control, latrunculin A-, and jasplakinolide-treated cells. The results shown in Figure 6 demonstrate that the levels of both exogenous and endogenous full-length actin mRNA were elevated in jasplakinolide-treated cells (Fig. 6A, lane 4; 6B, lane 3) and reduced in latrunculin A-treated cells (Fig. 6A, lane 3; 6B, lane 2), as compared with control (Fig. 6A, lane 2; 6B, lane 1). By contrast, the level of actin mRNA lacking the 3′-UTR and that of the control Neo^r remained unchanged after treatment with these drugs (Fig. 6B, lanes 5–7). These results indicate that deletion of the 3′-UTR in actin mRNA rendered β-actin mRNA insensitive to the G-actin-inducible regulation of its level, and that the 3′-UTR of actin mRNA is necessary for both the increase and the de-

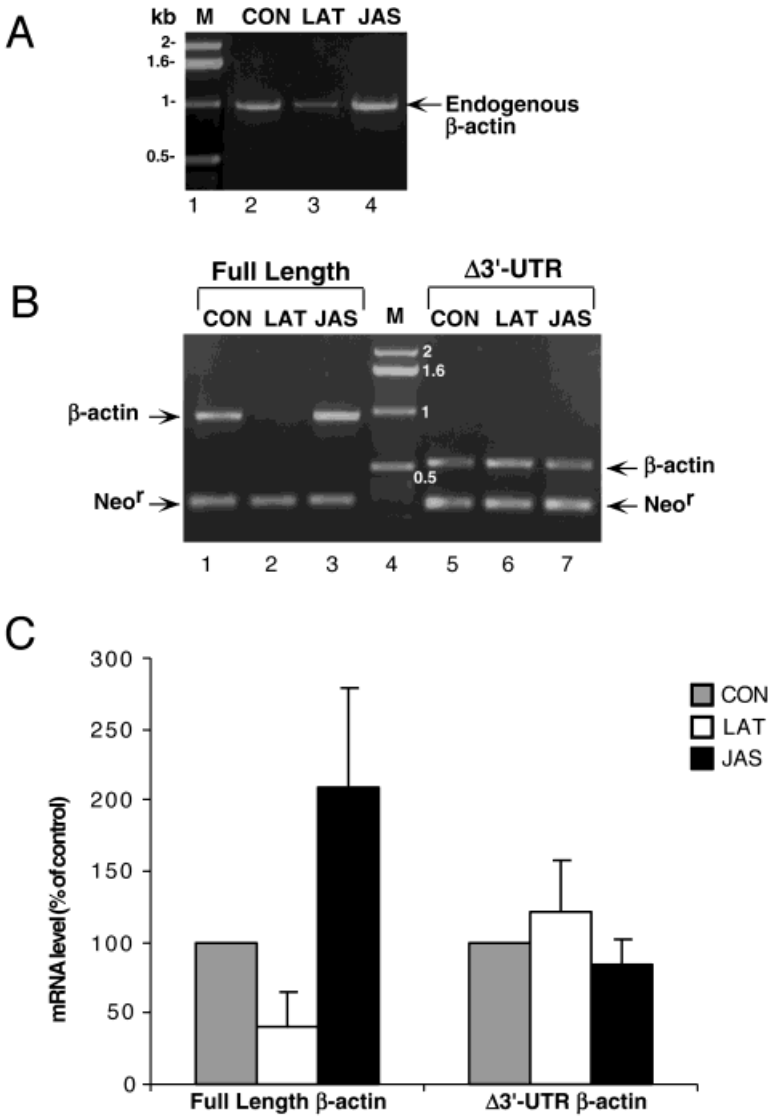

Fig. 6. Effects of latrunculin A and jasplakinolide on the level of full-length and 3′-UTR-deficient β-actin mRNA. Level of endogenous **(A)** β-actin, and (**B**) transfected full-length (FL) and 3′-UTR-deficient (Δ3′-UTR) β-actin mRNA were determined by reverse transcription-polymerase chain reaction (RT-PCR) in untreated cells (CON) (**A**, **lane 2**; B, **lanes 1, 5**), and in cells treated with latrunculin A (LAT) (**A**, **lane 3**; **B**, **lanes 2, 6**), or jasplakinolide (JAS) (**A**, **lane 4**; **B**, **lanes 3, 7**). **C:** PCR products corresponding to actin and the Neo^r gene were quantified by densitometry. The levels of actin mRNA were normalized to that of the Neo^r gene. The effects of latrunculin A and jasplakinolide treatment are presented as a percentage of control. The data represent the mean ±SD from four (latrunculin A) and two (jasplakinolide) separate experiments.

crease in actin mRNA that is probably driven by the same autoregulatory mechanism.

Deletion of the 3′-UTR in β-Actin mRNA Increases the Level of Actin Expression and Changes Cell Morphology

To examine further the effect of the 3′-UTR in actin mRNA on the regulation of actin expression and possibly on the organization of microfilaments, we compared cells transfected with cDNA encoding 3′-UTR-containing versus 3′-UTR-deficient actin mRNA and analyzed the organization of actin filaments and the level of actin in these cells.

The transfected actin carried an N-terminal HA-tag enabling analysis of the expression of exogenous actin by Western blotting and visualization of its distribution in the transfected cells by immunofluorescence. To determine possible differences in HA-actin expression resulting from varying levels of plasmid transfection, the cells were co-transfected with different

amounts of HA-actin constructs and a GFP-encoding plasmid (Fig. 7A). The level of HA-actin and GFP were determined by Western blotting with anti-HA and -GFP antibodies and used for normalizing the amount of exogenous actin expressed (Fig. 7B).

Western blot analysis showed that SV-80 cells transfected with the 3′-UTR-deficient HA-actin expressed significantly higher levels of HA-actin than did cells transfected with equal amounts of 3′-UTR-containing HA-actin (Fig. 7A,B). The values ranged between 10- and 20-fold higher levels for HA-actin in cells transfected the 3′-UTR-deficient HA-actin, as compared with the level of HA-actin in the control cDNA-transfected cells (Fig. 7B).

The characteristic effects on cell morphology and actin organization in cells overexpressing control or 3′-UTR-lacking HA-actin constructs are shown in Figure 8C,D and E,F, respectively. The predominant morphology of cells expressing 3′-UTR-containing HA-actin was similar to that of nontransfected cells and included spread cells that displayed actin stress fibers (Fig. 8C,D). When compared with nontransfected cells, these cells displayed more microspikes and F-actin containing ruffles on their periphery (Fig. 8C, cf. Fig. 8A). By contrast, cells transfected with the 3′-UTR-deficient HA-actin that expressed high levels of tagged actin displayed a dramatically different phenotype. In these cells, the development of stress fibers was strongly reduced and a significant part of actin was recruited to the cell periphery, where it formed numerous ruffles and filopodia-like structures (Fig. 8E,F, cf. Fig. 8A,C,D). These results suggest that expression of β-actin mRNA lacking the 3′-UTR leads to actin overproduction, abnormal organization of the actin cytoskeleton, and alteration in cell morphology.

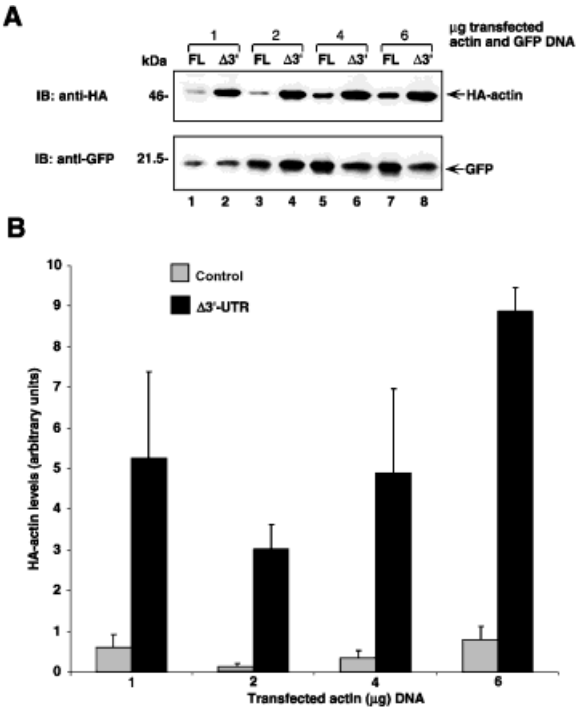

Fig. 7. The levels of transfected control and 3′-UTR-deficient HA-tagged actin constructs are different in SV-80 cells. **A:** SV-80 cells transfected with 1 μg (**lanes 1, 2**), 2 μg (**lanes 3, 4**), 4 μg (**lanes 5, 6**) and 6 μg (**lanes 7, 8**) control, or 3′-UTR-lacking (Δ3′) HA-actin constructs together with the same increasing amounts of GFP were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting with antibodies to the HA-tag and GFP. **B:** HA-actin expression was quantified by densitometry and normalized to the level of GFP. Bars represent the mean ±SD from three independent determinations.

DISCUSSION

Previous studies have demonstrated that the rate of β-actin expression inversely correlates with the level of G-actin in the cytoplasm. This feedback regulation operates post-transcriptionally, involving changes in actin mRNA stability [Bershadsky et al., 1995; Lyubimova et al., 1997]. Several examples of negative feedback, linking the level of the protein and the stability of its mRNA, have been described for proteins other than actin. Depolymerization of tubulin was shown to result in the specific destabilization of β-tubulin mRNA [Cleveland, 1988; Theodorakis and Cleveland, 1992]. The regulation of histone synthesis including a link between the level of histone-protein and the stability of its mRNA, is another example for this type of regulation [Peltz and Ross, 1987]. The mechanisms underlying such type of autoregulation are different in each case. A common feature however, shared by these mRNA is a sequence that renders the mRNA molecule a target for a regulated degradation. Deletions or mutations in this sequence, which is localized in different parts of the mRNA molecule, disrupt the autoregulation-linked degradation of these mRNA [Luscher et al., 1985; Gay et al., 1987; Bachurski et al., 1994]. For example, the sequence linking β-tubulin mRNA stability to the level of nonpolymerized β-tubulin subunits is confined to the first 13 nucleotides of its coding region [Gay et al., 1987; Bachurski et al., 1994], while the sequence required for histone mRNA stabil-

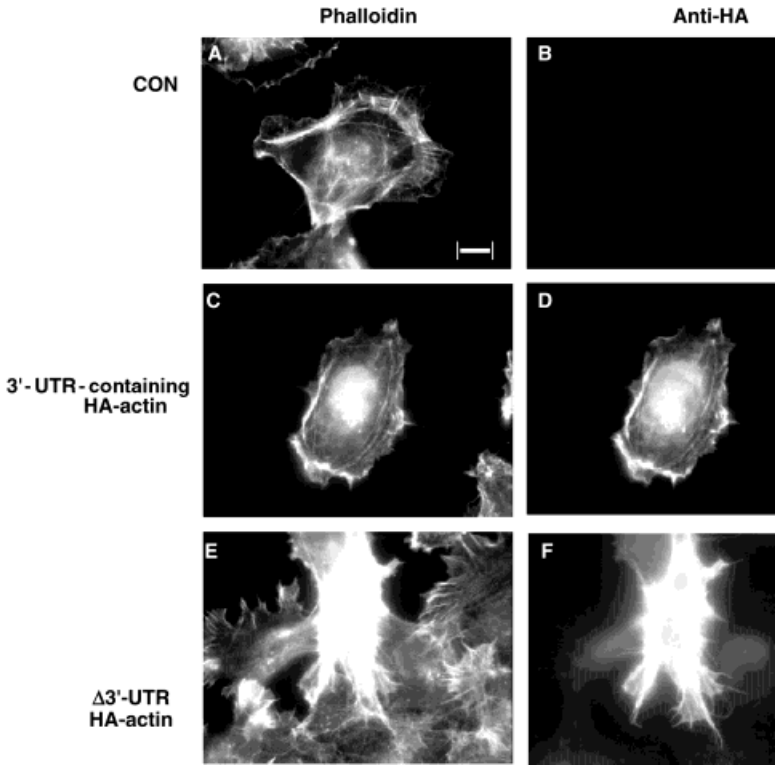

Fig. 8. SV-80 cells expressing 3′-UTR-containing and 3′-UTR lacking HA-actin constructs are morphologically different. Untransfected (**A,B**) and transiently transfected cells with 3′-UTR-containing (**C,D**) or 3′-UTR-deficient (**E,F**) HA-actin cDNA were fixed 30 h after transfection and double-stained with anti-HA-tag antibody to visualize the transfected actin (**B,D,F**) and with FITC-phalloidin (**A,C,E**) to visualize polymerized actin. Scale bar = 10 μm.

ity regulation is localized at the terminal 80 nucleotides of its 3′-UTR [Stauber et al., 1986].

By analyzing the effects of the changes in G-actin concentration on the level of β-actin mRNA displaying various deletions, we demonstrated that the 3′-UTR of β-actin mRNA contains sequences that are necessary for the G-actin-dependent control of mRNA stability. The level of full-length actin mRNA in the cell was reduced upon actin depolymerization by latrunculin A and was elevated when G-actin was sequestered by jasplakinolide. By contrast, the level of actin mRNA lacking the 3′-UTR remained unchanged by modulations in actin assembly by these drugs (Fig. 9).

The details of the molecular mechanism by which the 3′-UTR of actin mRNA participates in the G-actin-mediated regulation of actin mRNA stability remain to be determined. This mechanism may resemble the one operating in other mRNA where the 3′-UTR of the mRNA contains binding sites for proteins that can directly affect its stability [Ross, 1995]. Such putative actin mRNA-protein interactions may depend on G-actin that by associating with a protein complex affecting mRNA stability, could promote the specific degradation of actin mRNA by recruiting, for example, endonucleases to this complex.

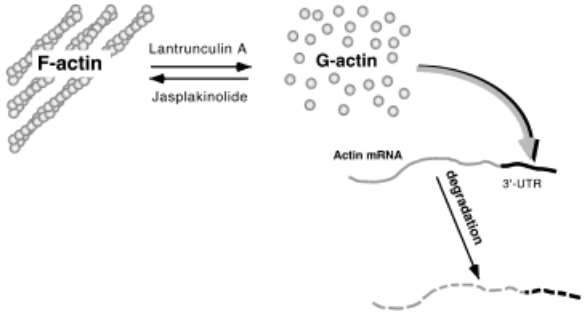

Fig. 9. Proposed model for the autoregulation mechanism controlling actin synthesis. In this model, G-actin is suggested to promote actin mRNA degradation in a process involving the 3′-UTR of actin mRNA. An increase in the pool of G-actin induced by latrunculin A will result in decreased actin synthesis, owing to destabilization of actin mRNA, whereas the reduction in the G-actin level through its conversion to F-actin by jasplakinolide treatment will result in increased actin synthesis.

It is noteworthy that the 3′-UTR of β-actin mRNA contains a conserved sequence, the "zipcode" [Kislauskis et al., 1994], that is responsible for the proper localization of β-actin mRNA to the actin filament network at the leading edge of migrating fibroblasts [Kislauskis et al., 1993, 1994]. Recently, a 68-kDa zipcode binding protein (ZBP-1) was isolated [Ross et al., 1997] and found to be homologous to *Xenopus* Vera, or Vg RPB, which binds to a similar sequence at

the 3′-UTR of *Vg1* mRNA determining its localization to microtubules [Deshler et al., 1998; Havin et al., 1998]. This finding suggests that the autoregulation of actin expression may depend on the localization of its mRNA. Localization-dependent regulation of mRNA stability was already described for histone mRNA [Zambetti et al., 1990]. On the other hand, the correct localization of actin mRNA could be driven by a selective degradation of actin mRNA molecules that are localized at inappropriate sites in the cell. The autoregulatory mechanism that controls mRNA stability may thus serve to degrade mislocalized mRNA selectively.

In most studies on the actin assembly-dependent regulation of actin expression and similarly in the studies on tubulin autoregulation, the level of actin and tubulin assembly in the cell was modulated using a variety of drugs. The microtubule-depolymerizing toxins colchicine and nocodazole and the microtubule-stabilizing/sequestering drugs taxol and vinblastine were employed in experiments on tubulin autoregulation [Ben-Ze'ev et al., 1979; Cleveland et al., 1981]. In studies on actin autoregulation, latrunculin A and *Clostridium botulinum* C2 toxin [Aktories et al., 1986] were applied to increase the level of G-actin; F-actin stabilizing agent phalloidin and swinholide A that forms actin dimers were also used [Serpinskaya et al., 1990; Reuner et al., 1991; Bershadsky et al., 1995; Reuner et al., 1995b; Lyubimova et al., 1997].

In this work, the question of the biological significance of the actin monomer-mediated actin mRNA turnover was addressed by transfection of cDNA encoding actin mRNA lacking the 3′-UTR. Transient expression of the autoregulation-deficient mRNA revealed that the actin mRNA that was released from the negative feedback control accumulated to high levels and, consequently, a dramatic increase in actin production ensued. The level of actin in cells transfected with constructs lacking the 3′-UTR of the mRNA was 10- to 20-fold higher than in cells transfected with the control cDNA. Analysis of the morphology of such cells demonstrated that excessive actin expression correlated with abnormalities in actin cytoskeleton organization and cell shape. These results strongly suggest that the G-actin-dependent regulation of actin synthesis may serve to maintain a constant pool of actin monomers in cells and that the autoregulation mechanism is required for the proper organization of the actin cytoskeleton.

Interestingly, a recent study has demonstrated a central role for the level of G-actin also in the transcriptional regulation of a subset of serum responsive factor (SRF) target genes, including the actin and vinculin genes [Sotiropoulos et al., 1999]. In analogy to the present study, monomeric (but not polymeric) actin was implicated in the regulation of signaling that leads to the transcriptional activation of this subset of immediate early genes induced by serum via SRF. It is possible that cells employ the monitoring of monomeric actin level as a critical regulator of signaling, at both the transcriptional and post-transcriptional levels, to transduce signals that are conveyed by changes in the assembly of the cytoskeleton [Sotiropoulos et al., 1999].

In the present study, the level of actin mRNA and the amount of monomeric actin subunits were linked via a sequence localized in the 3′-UTR of the actin mRNA. Future studies will have to determine the minimal sequence(s) in the 3′-UTR of the actin mRNA involved in this autoregulation and the factors interacting with such sequence(s) to regulate in concert with G-actin the stability of β-actin mRNA.

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