β-Actin mRNA Localization Is Regulated by Signal Transduction Mechanisms

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Abstract. β-actin mRNA is localized in the leading lamellae of chicken embryo fibroblasts (CEFs) (Lawrence, J., and R. Singer. 1986. *Cell.* 45:407–415), close to where actin polymerization in the lamellipodia drives cellular motility. During serum starvation β-actin mRNA becomes diffuse and non-localized. Addition of FCS induces a rapid (within 2–5 min) redistribution of β-actin mRNA into the leading lamellae. A similar redistribution was seen with PDGF, a fibroblast chemotactic factor. PDGF-induced β-actin mRNA redistribution was inhibited by the tyrosine kinase inhibitor herbimycin, indicating that this process requires intact tyrosine kinase activity, similar to actin filament polymerization and chemotaxis. Lysophosphatidic acid, which has been shown to rapidly induce actin stress fiber formation (Ridley, A., and A. Hall. 1992. *Cell.* 790:389–399), also increases peripheral β-actin mRNA localization within minutes. This suggests that actin polymerization and mRNA localization may be regulated by similar signaling pathways. Additionally, activators or inhibitors of kinase A or C can also delocalize steady-state β-actin mRNA in cells grown in serum, and can inhibit the serum induction of peripherally localized β-actin mRNA in serum-starved CEFs. These data show that physiologically relevant extracellular factors operating through a signal transduction pathway can regulate spatial sites of actin protein synthesis, which may in turn affect cellular polarity and motility.

I N chicken embryo fibroblasts (CEFs)[1] β-actin mRNA is localized near the leading edge of the cell in a region referred to as the lamella, in contrast to other specific mRNAs such as that for tubulin or vimentin (Lawrence and Singer, 1986), or total cellular mRNA, as visualized with probes for poly(A) (Taneja et al., 1992). Sorting of actin mRNAs appears to be highly specific, since mRNA for different isoforms of actin have been shown to be differentially localized within a common cytoplasm (Hill and Gunning, 1993; Kislauskis et al., 1993). While the precise role of actin mRNA localization is not defined, a growing body of evidence showing intracellular sorting of mRNAs in a variety of cell types indicates that modulation of the sites of synthesis of specific proteins is an important component of gene expression (for review see Kislauskis and Singer, 1992; Wilhelm and Vale, 1993). In cells such as fibroblasts, which respond to chemotactic stimulation, actin filament formation in the leading lamella plays a vital role in the extension of the leading edge during cell motility (Wang, 1984). It has been suggested that the asymmetric distribution of β-actin mRNA functions to support the polarity of the cell, through

restricted spatial distribution of actin protein synthesis, which is necessary for directional movement (Lawrence and Singer, 1986).

How cells regulate mRNA localization is not known. Peripheral localization of β-actin mRNA does not require ongoing protein synthesis (Sundell and Singer, 1990) and is mediated by an interaction between β-actin mRNA and the actin-based cytoskeletal framework (Sundell and Singer, 1991). Since signal transduction mechanisms mediate the functionally related events of growth factor stimulation; cytoskeletal reorganization and directional cellular motility (Ullrich and Schlessinger, 1990; Cross and Dexter, 1991; Schlessinger and Ullrich, 1992), the localization of β-actin mRNA may also be responsive to these signals.

Maintenance of the normal growth and morphology of cells in culture is dependent on the continued presence of serum in the medium, in part due to the presence of serum growth factors (Ross et al., 1974; Pledger et al., 1982). Serum starvation induces cells to enter a quiescent phase of the cell cycle (Pardee, 1974), which is characterized by a decrease in the expression of the β-actin gene (Pardee et al., 1978). Serum starvation also causes a reduction in the number of actin stress fibers, focal adhesions, and a decrease in the amount of polymerized actin in the cell periphery (Ridley and Hall, 1992). Subsequent reintroduction of either serum or of growth factors such as PDGF, results in the transient expression of cytoskeletal actin genes (Greenberg and Ziff,

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1. *Abbreviations used in this paper:* CEF, chicken embryo fibroblast; LPA, lysophosphatidic acid; TPA, 12-o-tetradecanoyl-phorbol-13-acetate.

1984) and is accompanied by a marked increase in polymerized actin (Bockus and Stiles, 1984; Herman and Pledger, 1985).

The mechanism by which growth factors induce cell proliferation and chemotaxis is becoming more defined. For PDGF, a potent fibroblast proliferative (Pledger et al., 1982) and chemotactic factor (Seppa et al., 1982; Senior et al., 1983), receptor binding is followed by dimerization, activation of tyrosine kinase activity and subsequent generation of second messenger molecules that propagate the cellular responses (see Heldin and Westermark, 1990). Recent work suggests that the ras protein acts as a transducer of the PDGF-receptor–mediated signaling pathway (Satoh et al., 1990, 1993), accounting for both increased proliferation and chemotaxis through divergent downstream pathways. PDGF can induce changes in the actin cytoskeleton through the activation of the PDGF receptor, which results in the activation of ras and the subsequent activation of rho and rac, resulting in the formation of peripheral actin filaments and actin stress fibers (Ridley and Hall, 1992; Ridley et al., 1992). Additionally, other PDGF-associated signaling molecules, such as phospholipase C-γ, have been shown to be associated with membrane ruffles and actin filaments (Bar-Sagi et al., 1993). These growth factor induced changes in the actin cytoskeleton may then allow for increased cellular motility. Our data indicate that these same signal transduction pathways operate in regulating β-actin mRNA localization.

The linkage of mRNA localization with a growth factor receptor would be an important advance in understanding both the physiological significance and the mechanism of this phenomenon. This would provide a means for the cell to respond to growth factors by modeling gene expression to reorient sites of synthesis of specific proteins. The regulation of β-actin mRNA localization by growth factors may also be important in the maintenance of cellular polarity and directional motility.

Materials and Methods

Culture and Fixation of Chick Embryo Fibroblasts

Fibroblast cells were isolated from breast muscle tissue of 12-d chick embryos as described (Lawrence and Singer, 1986). Cells were plated onto either 10-cm culture dishes or gelatin-coated coverslips at a density of 6 × 10^5 cells/ml. Cultures were grown at 37°C in MEM supplemented with 10% FCS in an atmosphere of 95% air/5% CO_2. For serum starvation, cells were grown to 60% confluence on coverslips and incubated in DME for 24 h. For in situ hybridization cells were grown on coverslips for 2 d and were fixed in 4% paraformaldehyde in PBS for 10 min at room temp following growth factor or phamacological treatments (see below). Cells were stored in 70% ethanol at 4°C for several days, and then processed for in situ hybridization.

Growth Factor and Pharmacological Treatments

CEF cultures were grown to 60% confluence on coverslips and either used directly or serum starved for 24 h. Before treatment cultures were rinsed in HBSS and incubated in MEM with or without (indicated in figure legend) 10% FCS supplemented with the given pharmacological agent. After treatment coverslips were washed in HBSS, fixed in 4% paraformaldehyde, stored in 70% ethanol, and hybridized in situ using β-actin oligonucleotide probes as described below. PDGF (human recombinant AB isoform; Upstate Biotechnologies Inc., Lake Placid, NY) was obtained as a stock of 65 μg/ml in 20 mM Tris/0.3M NaCl and diluted to a working stock of 6.5 μg/ml in 4 mM HCl with 1% BSA. Lysophosphatidic acid (LPA; Oleoyl-sn-glycero-3-phosphate; Sigma Chemical Co., St. Louis, MO) was prepared as a 10 mg/ml stock in a solution of 95% chloroform, 5% methanol, 5%

glacial acetic acid, and diluted in water. Forskolin and Forskolin, 1, 9-Dideoxy (Calbiochem-Behring Corp., San Diego, CA) were prepared as a 10 mM stock in DMSO. 12-o-tetradecanoyl-phorbol-13-acetate (TPA; Sigma Chemical Co.) and 4-phorbol 12,13 diacetate (Sigma Chemical Co.) were prepared as 10 mM stocks in DMSO. Herbimycin (Calbiochem-Behring Corp.) was prepared as a 1.75 mM stock in DMSO. Cordycepin (Calbiochem-Behring Corp.) was prepared as a 10 mg/ml stock in DMSO. K-252b (Calbiochem-Behring Corp.) was prepared as a 1 mg/ml stock in DMSO.

Detection of β-Actin mRNA by In Situ Hybridization

Probes. Oligonucleotide probes were prepared as described (Taneja and Singer, 1990). Eight oligonucleotide probes specific to chick β-actin mRNA were synthesized and end labeled with digoxigenin-dUTP. Digoxigenin-labeled nick-translated probes were prepared as described (Sundell and Singer, 1990). The cDNA probe used was a 1.38-kb segment (nucleotide 95-1477) of a full-length β-actin cDNA probe (Cleveland et al., 1980). For each coverslip, 5 ng of each oligonucleotide (40 ng total), or 20 ng for the nick-translated probe, were lyophilized with 40 μg Escherichia coli tRNA and 40 μg sonicated salmon sperm DNA for the oligonucleotide probes and 20 μg of each for the nick-translated probe. Dried probes were resuspended in 15 μl 100% formamide, and combined with 15 μl hybridization buffer (4× SSC, 0.4% BSA, and 20% dextran sulfate).

In Situ Hybridization. Cells stored in 70% ethanol at 4°C were rehydrated with PBS supplemented with 5 mM $MgCl_2$ and 0.5% Triton X-100 for 10 min. Prior to hybridization, coverslips containing cells were incubated in 50% formamide, 2× SSC and 5 mM NaH_2PO_4, pH 7.0 for 10 min at room temp. The coverslips were placed cell side down on parafilm containing 30 μl of formamide/hybridization buffer containing the appropriate probes, and incubated overnight at 37°C in a humidified chamber. After hybridization, coverslips were washed for 30 min at 37°C in 10 ml coplin jars containing 50% formamide, 2× SSC, followed by three 10-min washes in 1× SSC at room temp with gentle agitation. After equilibration in buffer 1 (150 mM NaCl and 100 mM Tris-Cl, pH 7.5) for 1 min, cells were incubated in 30 μl of buffer 1 plus 1% BSA containing a 1:250 dilution of anti-digoxigenin/alkaline phosphatase conjugate (Boehringer Mannheim Biochemicals, Indianapolis, IN) at 37°C for 30 min. Cells were washed twice for 15 min at room temperature in buffer 1 followed by equilibration in buffer 3 (100 mM Tris-Cl, pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$) for 1 min at room temp. Cells on coverslips were then transferred to a six-well tissue culture dish containing 2 ml of nitroblue tetrazolium/bromochloro-indolyl phosphate solution (3 mg NBT and 2 mg BCIP in 10 ml of buffer 3). The color development was monitored at room temperature using a water immersion objective and stopped by the addition of water when sufficient reaction product was observed (30–60 min).

Quantitation of β-Actin mRNA Localization

After in situ hybridization, coverslips were viewed by bright field microscopy and scored for the percentage of cells showing β-actin mRNA localization. Cells were scored as having localized β-actin mRNA if at least 80% of the signal was concentrated over leading lamellae, an area comprising ∼25% of the cell area. Random fields of cells under low power were selected, from at least two separate coverslips per condition, and all cells in the field were scored. Approximately 400 cells per coverslip were counted. Replicates were averaged and data plotted as percent of cells showing peripheral localized β-actin mRNA or converted to a ratio of the percent of treated cells showing localized mRNA to untreated controls. One way analysis of variance (ANOVA) was used to determine the statistical significance of the treated vs. control cells. Additionally Tukey HSD analysis (Miller, 1981) was used to determine significance of each concentration of each treatment.

Isolation of RNA and Northern Blotting

Total cellular RNA was isolated from confluent 10 cm plates of CEFs following the method of Xie and Rothblum (1991). Cells were rinsed with PBS and lysed directly on the plates with 1.6 ml of a solution of (water-saturated phenol, 4 M guanidinium thiocyanate, 25 mM sodium citrate pH 7.0, and 2 M NaOAc, pH 4.0, in a ratio of 1:1:1:0.1) plus 720 μl of β-mercaptoethanol per 100 ml. The lysate was mixed, and combined with 0.16 ml of chloroform/isoamyl alcohol (24:1) and vortexed. The solution was kept on ice for 15 min, centrifuged at 12,000 g for 20 min at 4°C, and RNA was precipitated from the aqueous phase with an equal volume of isopropanol at −20°C for 1 h. Agarose gel electrophoresis and Northern blotting was car-

ried out using standard procedures. β-actin mRNA was detected with β-actin cDNA labeled with digoxigenin by random priming (Boehringer Mannheim Biochemicals).

Detergent Extractions

10-cm plates of semi-confluent cultures were rinsed with PBS and then 1 ml of cold CSK buffer (0.3 M sucrose, 0.1 M KCl, 5 mM MgCl₂, 10 mM Pipes, pH 6.9, 2 mM EGTA, 2 mM PMSF, 2 mM vanadyl complex, 1 mg/ml leupeptin, and 1 mg/ml trypsin inhibitor) for 1 min. The cells were then exposed to 1 ml CSK supplemented with 0.5% Triton at 4°C for the indicated time. This extraction buffer was then removed (extracted fraction) and the remaining cytoskeletal material scraped in 0.5 ml CSK buffer plus triton (retained fraction). The extracted and retained fractions were vortexed, extracted on ice for 15 min, and clarified by centrifugation at 5000 g for 30 s. The supernatants were phenol extracted, ethanol precipitated, and the RNA pellet rinsed with 70% ETOH. Agarose gel electrophoresis and Northern blot detection was performed as described above.

Results

Serum, PDGF and LPA Induce β-Actin mRNA Localization

To determine pathways affecting mRNA localization the distribution of β-actin mRNA was monitored as a function of stimulation with serum, growth factors, and treatment with pharmacological agents which act on these signaling mechanisms. In situ hybridization with specific DNA probes showed that in CEFs cultured under normal growth conditions (10% FCS), β-actin mRNA was highly localized to the cell periphery in ∼30–35% of the cells (see Fig. 2, A, B, and D, solid circles). That 30–35% of cells in a population had localized mRNA at any given time, may have resulted from a steady-state equilibrium between localized and nonlocalized mRNA. Supporting this are our observations (below) that the percentage of cells scored as having localized β-actin mRNA can be significantly increased with long-term exposure to chemotactic factors and significantly decreased by serum starvation.

To first establish a baseline of localization in the absence of serum, cells were incubated in serum-free DME for 24 h and β-actin mRNA distribution monitored (Fig. 1 A). Serum starvation reduced the total percentage of cells exhibiting peripheral β-actin mRNA approximately threefold to 12.5% of total (Figs. 1 A and 2, A, B, and D). These cells appeared to be less polarized than cells grown continuously in serum (Fig. 1, A and E). Readdition of MEM containing 15% FCS resulted in a marked increase in cells with peripheral β-actin mRNA (Fig. 1, B–D). An increase was noted after 1–2 min, and at 10 min after serum addition the percentage of cells exhibiting peripheral β-actin mRNA had approximately doubled, to 20–25% of total (Fig. 2 B). By 3 h after serum addition, the level of β-actin mRNA localization approached that of normal cells (Fig. 2 B).

Serum starvation is known to cause cells to enter a quiescent phase of the cell cycle and to reduce β-actin gene expression (Greenberg and Ziff, 1984), and consequently results in a lowered steady-state level of mRNA. To determine if the localization of mRNA by serum stimulation represented newly synthesized mRNA, levels of total cellular β-actin mRNA were monitored throughout the serum starvation and stimulation. Northern analysis showed that after 24 h in serum-free conditions the level of cytoplasmic

β-actin mRNA was ∼37% of control levels (Fig. 3, lanes 1 and 2). Readdition of 15% serum resulted in an increase in β-actin mRNA levels (Fig. 3). However, no significant increase in cytoplasmic β-actin mRNA was seen until 60 min after serum stimulation (Fig. 3, lane 4), indicating that the observed changes in β-actin mRNA distribution, which occurs within 2–5 min, were due to relocalization of preexistent diffusely distributed mRNA.

FCS is known to contain a variety of growth and chemotactic factors, including PDGF (Balk, 1971; Kohler and Lipton, 1974). Since peripheral β-actin mRNA is thought to play a role in cellular motility the effect of PDGF on β-actin mRNA distribution was examined. Addition of purified PDGF induced a dose dependent increase in the percentage of CEFs exhibiting peripheral β-actin mRNA localization (Fig. 2 C). The time-course of the PDGF effect was examined using serum-starved cells. Addition of PDGF (50 ng/ml) to serum-starved CEFs resulted in a rapid increase in percent of cells exhibiting peripherally localized β-actin mRNA (Fig. 2 A). While the time course of the PDGF-induced increase in peripheral β-actin mRNA localization was somewhat slower than that seen with serum, an increase was seen as early as 5–10 min after treatment, and was greater than 2.5-fold at 180 min (Fig. 2, A and B). The effect seen with serum-starved cells may exceed that seen in normal cells because the baseline of localization is considerably lower, and thus a larger fold effect is possible. As with serum, the short time of the response to PDGF suggests that the increase in peripheral β-actin mRNA results from a relocalization of preexistent, diffusely distributed mRNAs.

Since PDGF may mediate changes in the actin cytoskeleton through activation of the ras family of GTPases (Ridley and Hall, 1992), the influence of activation of these pathways on β-actin mRNA distribution was examined. LPA is a potent stimulator of platelet aggregation which has been shown to activate the rho-class of GTPases (Tsai et al., 1989; Ridley and Hall, 1992). Addition of LPA to serum-starved CEFs resulted in a significant increase in cells exhibiting peripheral β-actin mRNA (Fig. 2 D). The LPA induced increase in β-actin mRNA localization occurs along the same immediate time course as that of PDGF, with significant increases seen at 5 and 10 min after treatment, but unlike PDGF, the effect diminishes after 10 min (Fig. 2, A and D).

Phosphorylation Pathways Affect β-Actin mRNA Localization

It has been shown that the intrinsic tyrosine kinase activity of the PDGF receptor is essential for stimulation of mitogenesis (Escobedo et al., 1988) and chemotaxis (Westermark et al., 1990). To determine if the signaling pathway for mRNA localization also requires a functional tyrosine kinase, cells were exposed simultaneously to PDGF and herbimycin, an inhibitor of tyrosine phosphorylation (Uehara et al., 1986), and β-actin mRNA distribution was monitored. As shown in Fig. 4 B, herbimycin inhibited the PDGF-induced increase in mRNA localization ($P = 0.031$). Moreover, herbimycin inhibited the steady-state localization significantly ($P = 0.02$) (Fig. 5 E), and also was the most effective inhibitor of serum stimulation ($P = 0.021$) (Fig. 4 A). This suggests that localization of β-actin mRNA requires an intact tyrosine kinase to induce and maintain localization, and that growth
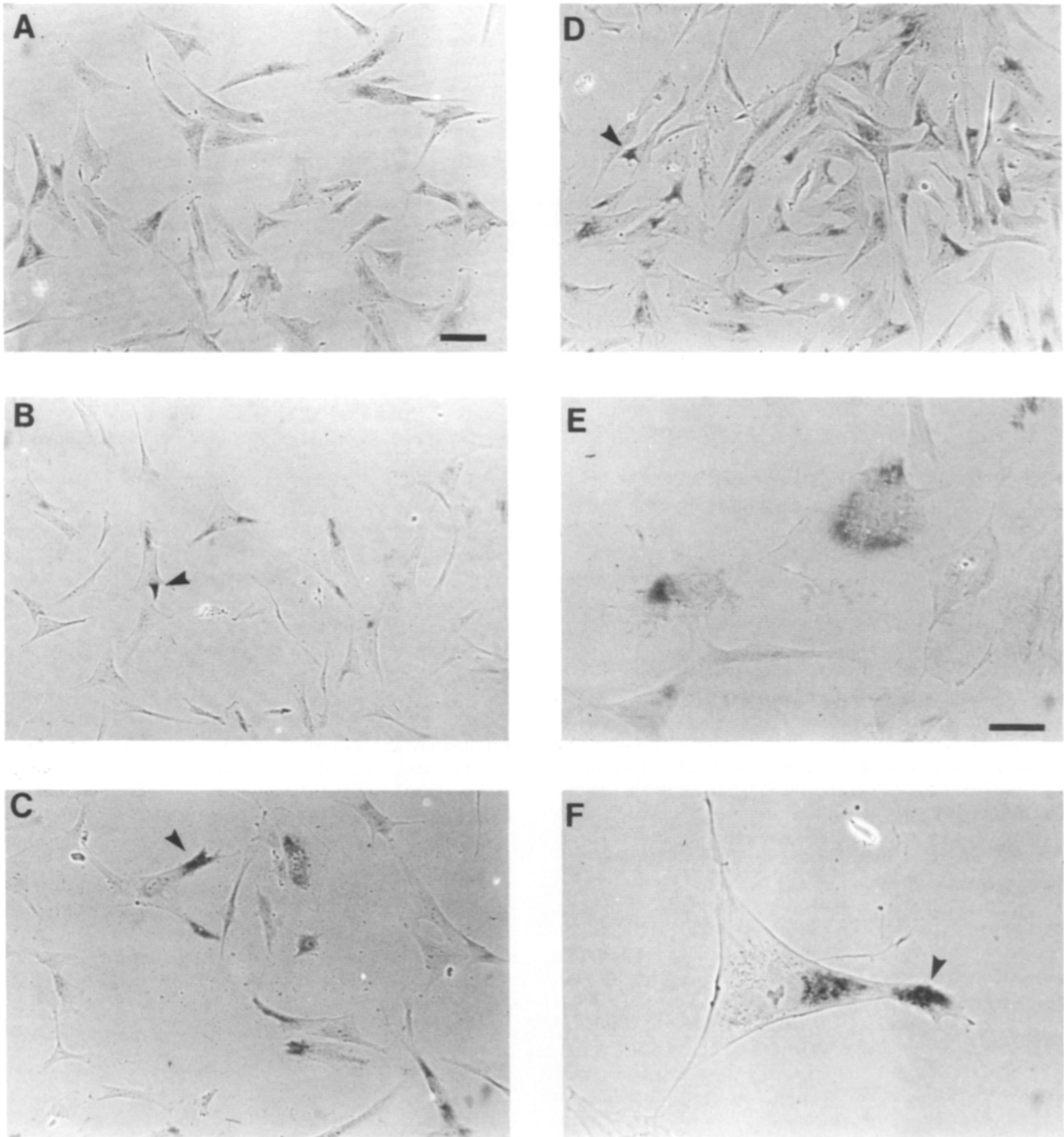
Figure 1. The effect of serum starvation and stimulation on β-actin mRNA localization. CEFs were serum starved for 24 h and stimulated with 15% FCS in MEM for 0 (*A* and *E*), 30 min (*B* and *F*), 60 min (*C*), or 6 h (*D*). β-actin mRNA was visualized by in situ hybridization as described in Materials and Methods using nick-translated probes. Note the relatively diffuse staining in the serum-starved cells (*A*), and the gradual increase in localized mRNA and number of cells with peripherally localized β-actin mRNA (some of which are arrowed) in the serum-stimulated cells (*B–D*). *E* and *F* show higher magnification images of cells serum starved (*E*), and stimulated for 30 min after serum starvation (*F*); note the diffuse staining in *E* and the shift to lamellar staining in *F* (*arrow*). Bars, (*A–D*) 25 μm, (*E* and *F*) 10 μm.

factor stimulation of localization occurs through a tyrosine kinase signal transduction pathway.

To determine if other intracellular phosphorylation pathways influence normal peripheral β-actin mRNA localization, CEFs were treated with a variety of well characterized kinase activators or inhibitors and β-actin mRNA distribution was monitored. TPA, a potent activator of protein kinase C (Castagna et al., 1982), induced a rapid (within 15 min), dose-dependent delocalization of β-actin mRNA ($P = 0.02$, Fig. 5 *B*). In contrast, 4-phorbol 12,13 diacetate, which is
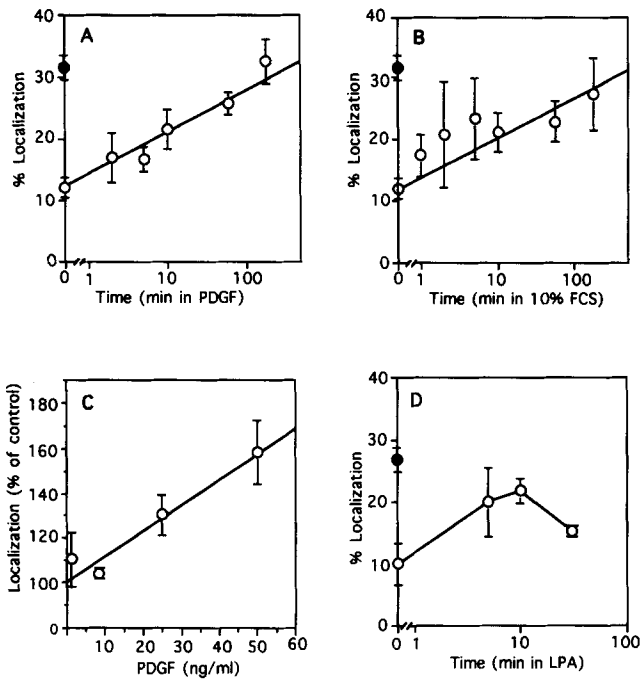
Figure 2. Stimulation of β-actin mRNA localization. Serum-starved CEFs were treated with PDGF (50 ng/ml) (*A*), or 10% FCS (*B*), or LPA (200 ng/ml) (*D*), for each time point. In *C*, PDGF treatment was for 18 h at each concentration on CEFs grown continuously in 10% serum. The β-actin mRNA localization was visualized and quantitated by in situ hybridization using β-actin oligonucleotide probes as described in Materials and Methods. Each data point represents mean plus or minus the standard error of at least two coverslips and for *A*, *B*, and *D*, and are plotted as the percentage of cells with peripherally localized β-actin mRNA vs. time. For *C*, the ratio of percentage of treated cells with peripherally localized β-actin mRNA to that of untreated controls is plotted vs. concentration. Solid circles (●) indicate the percentage localization of a parallel set of coverslips grown continuously in serum with no serum starvation.
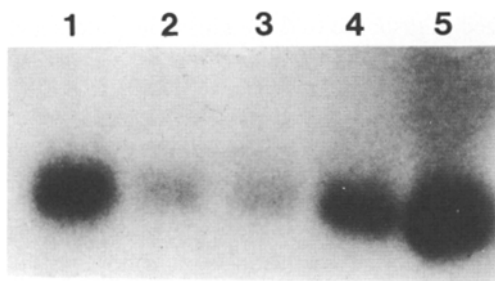

Figure 3. Northern blot analysis of β-actin mRNA levels in serum-starved and serum-stimulated cells. Confluent 10-cm plates of CEFs were serum starved as in Fig. 1, and β-actin mRNA quantitated by Northern blot as described in Materials and Methods. Bands representing β-actin were scanned and quantified on a β-scanner (Bio-Rad Laboratories, Cambridge, MA). Lane *1*, control cells not serum starved; lanes *2–5*, 24 h serum starvation with 0 min (lane *2*), 30 min (lane *3*), 60 min (lane *4*), and 6 h (lane *5*) after addition of 15% FCS. Ratios of bands are as follows: lanes *1*, 1.0; lane *2*, 0.37; lane *3*, 0.41; lane *4*, 0.90; lane *5*, 1.8. Positive pole is on the top.
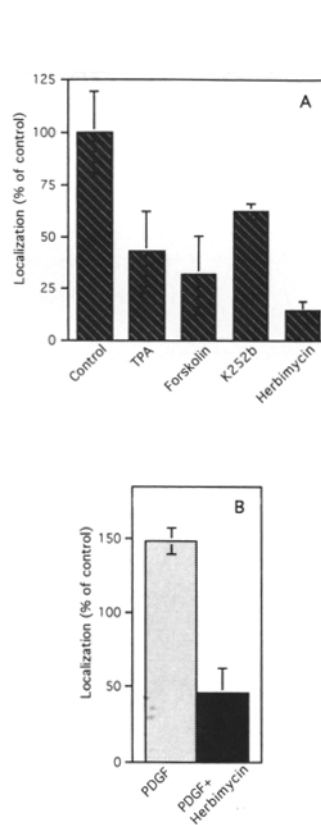

Figure 4. Effect of pharmacological agents on the induction of β-actin mRNA localization by serum and PDGF. (*A*) Serum-starved CEFs were incubated in 10% FCS/MEM for 90 min (*control*), and simultaneously treated with 1 μM TPA, 100 μM forskolin, 1 μM K252b, and 875 nM herbimycin before fixation. β-actin mRNA localization was visualized and quantitated by in situ hybridization, using β-actin oligonucleotide probes as described in Materials and Methods. Each data point represents the mean plus or minus the standard error of at least two coverslips. (*B*) Effect of tyrosine kinase inhibitor herbimycin, on the PDGF-induced increase in β-actin mRNA localization. Serum-starved CEFs were treated for 3 h with 50 ng/ml PDGF alone, or PDGF and 875 nM herbimycin. Note that herbimycin completely abolishes PDGF-induced stimulation of β-actin mRNA localization.

structurally similar to TPA but biologically inactive (Blumberg, 1981), had little effect on β-actin mRNA distribution (*P* = 0.45, Fig. 5 *B*). In addition, activation of protein kinase A by forskolin also delocalized β-actin mRNA within 15 min (*P* = 0.016, Fig. 5 *A*), while an inactive analog, forskolin, 1, 9-dideoxy did not (*P* = 0.439, Fig. 5 *A*). K252b, an inhibitor of protein kinases C and A also delocalized β-actin mRNA (*P* = 0.026, Fig. 5 *C*). Finally, cordycepin (3'deoxyadenosine), which decreases cellular ATP levels (Zieve et al., 1987), rapidly delocalized β-actin mRNA (Fig. 5 *D*). These agents also inhibit the induction of β-actin mRNA localization in serum starved-cells after serum readdition for 90 min (Fig. 4 *A*). Both the delocalization of mRNA in cells grown in serum and the inhibition of mRNA localization after serum readdition occur at the same concentrations of these kinase inhibitors or stimulators (Figs. 4 *A* and 5, *A–E*). All of these data illustrate the acute sensitivity of β-actin mRNA localization to agents which alter cellular phosphorylation levels.

Localized and Delocalized β-Actin mRNA Are Bound to the Cytoskeleton

One model for the mechanism of action of the drugs which delocalize β-actin mRNA, would be a release of mRNA from its anchoring site and subsequent passive diffusion to a nonlocalized state. An established method for determining cytoskeletal associations involves treating cells with a mild, nonionic detergent and monitoring the partitioning between detergent soluble and insoluble phases (Lenk et al., 1977; Osborn and Weber, 1977; Taneja et al., 1992). Evidence from several other systems, including Vg 1 mRNA in oocytes
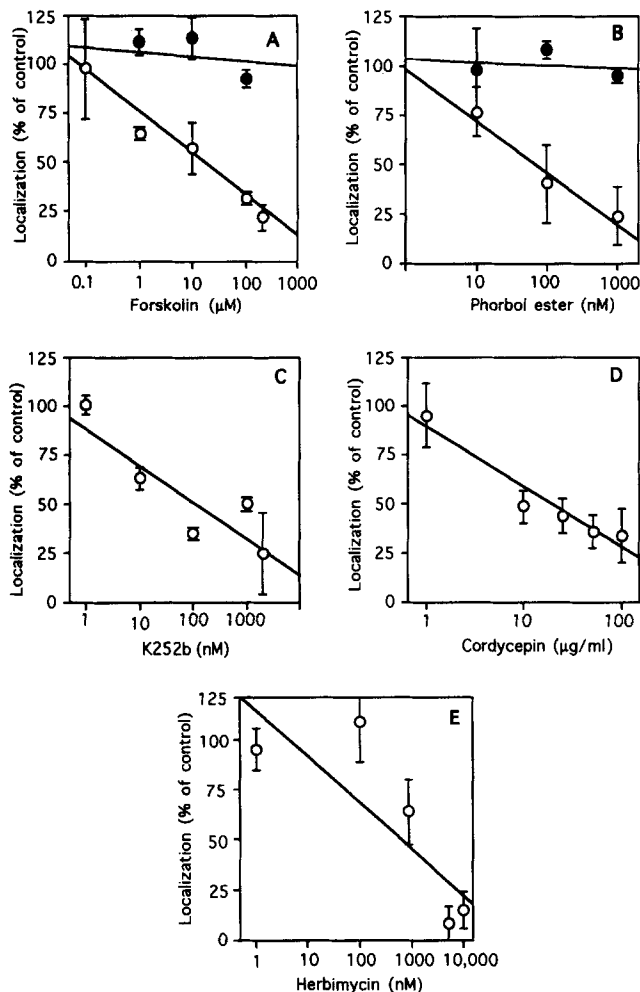
Figure 5. Effect of pharmocological agents on steady-state β-actin mRNA localization. Effect of forskolin (○), or forskolin, 1,9-dideoxy (●) (A); of TPA (○), or phorbol diacetate (●) (B); of K252b (C); of cordycepin (D); or herbimycin (E); treatment on β-actin mRNA localization. CEFs grown continuously in 10% FCS/MEM were treated with each agent at the given concentrations for 15 min (in 10% FCS/MEM), except herbimycin which was for 3 h. β-actin mRNA localization was visualized and quantitated by in situ hybridization, using β-actin oligonucleotide probes as described in Materials and Methods. Each data point represents mean plus or minus the standard error of at least two coverslips.

Figure 6. Detergent extraction of forskolin treated cultures. Confluent 10-cm plates of CEFs were treated with the either 100 μM forskolin (A), or the given concentration (B), and detergent extracted as described in Materials and Methods. (A) cultures were extracted for the times indicated and β-actin mRNA levels in the extracted and retained fractions were monitored by Northern blot analysis. (B) Dose–response of forskolin in cultures extracted for 1 min as described. Note that in every case the percentage of β-actin mRNA retained on the cytoskeletal framework is not significantly altered by forskolin.

(Melton, 1987), and myelin basic protein mRNA in oligodendrocytes (Ainger et al., 1993), indicates that localized mRNAs are selectively adherent to cytoskeletal structures while diffusely distributed mRNAs are readily extracted. If the kinase stimulating agents delocalize β-actin mRNA by releasing it from the cytoskeleton, an increase in the detergent extractability of β-actin mRNA would result. When fibroblasts with localized β-actin mRNA were detergent extracted, a majority of this mRNA remained associated with the cytoskeleton (Fig. 6), consistent with earlier reports (Bonneau et al., 1985; Singer et al., 1989). When cells were treated with forskolin under conditions which markedly delocalized β-actin mRNA, there was no change in the extractability of the mRNA relative to untreated cells (Fig. 6). It appears therefore, that actin mRNA remains associated
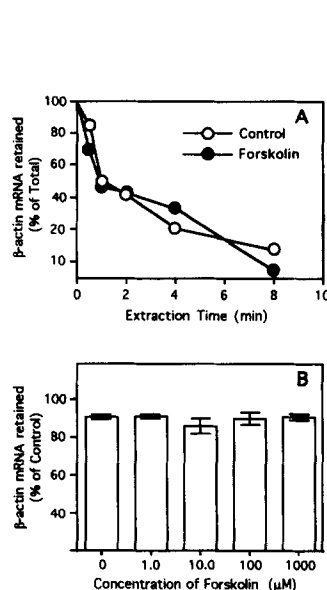
with the cytoskeleton even if it is no longer localized to one area of the cell.

Discussion

We have evaluated a mechanism by which extracellular factors influence intracellular β-actin mRNA trafficking in chick embryo fibroblasts. When cells were serum starved, β-actin mRNA became delocalized indicating a requirement for serum factors on continued localization. Consistent with this interpretation, when serum starved cells were stimulated with serum, PDGF, or LPA, β-actin mRNA moved rapidly from a diffuse distribution into a peripheral distribution in the lamella of the cell. Similar results have been obtained by Hill et al. (1994, in this issue). This movement of actin mRNA was prevented by kinase inhibitors and activators, indicating that the localization of β-actin mRNA responds to chemotactic factors through signal transduction pathways. Likewise β-actin mRNA which was already localized became delocalized in response to these kinase inhibitors and activators. A number of implications arise from these results. First, cytoplasmic actin mRNA can localize and delocalize rapidly. The localization from a delocalized state in response to growth factors takes only a few minutes, as does the delocalization in response to kinase activators and inhibitors. Second, this localization appears to be a target of the same mechanism which transduces extracellular signals into intracellular events. This mechanism may act through the ras GTPase system, in particular rho (or rac), and couple mRNA localization to changes in the actin cytoskeleton and lamellipodia extensions. Additionally, localization of β-actin mRNA may require a phosphorylation step in the pathway. The significance of these observations are that synthesis of a cellular structural protein can be controlled spatially by an extracellular signal through a signal transduction pathway.

The initial stage of this effect involves a redistribution of preexistent, diffusely distributed β-actin mRNA, rather than a selective localization of newly synthesized molecules. Although an increase in gene expression is evident within 5 min

of exposure to serum (Greenberg and Ziff, 1984), no increase in total cytoplasmic levels of β-actin mRNA was found until 60 min following stimulation of serum-starved cells. However, there was a reproducible and significant increase in β-actin mRNA localization within 5–10 min of exposure to either serum, PDGF, or LPA. This means that all of the components necessary for localization are present in the cytoplasm, precluding models of localization which require transcription to precede the localization. This is also supported by previous work showing that ongoing or steady-state localization in spreading cells is not affected by actinomycin, an inhibitor of transcription (Sundell and Singer, 1991).

How might chemotactic factors affect mRNA distribution? The induction of β-actin mRNA localization by PDGF and inhibition by herbimycin indicates that localization is a downstream event from both PDGF receptor activation and induction of its tyrosine kinase activity. This is consistent with receptor mutant studies which have shown that most cellular effects of PDGF, including actin reorganization and chemotaxis, require intact tyrosine kinase activity (Escobedo et al., 1988; Westermarch, 1990). One reported effect of PDGF and other growth factors is activation of ras-GTP (Gibbs et al., 1990; Satoh et al., 1990), which then initiates either cell division through activation of raf (Moodie et al., 1993) or chemotaxis through activation of rac and rho (as seen by increased actin polymerization, focal adhesions and stress fibers) (Ridley et al., 1992, Ridley and Hall, 1992). LPA can also induce activation of rho (Tsai et al., 1989). The ability of both LPA and PDGF to induce β-actin mRNA localization, suggests that this localization may possibly be through rho and rac as well. LPA induces β-actin mRNA relocalization, however, slightly faster than PDGF, peaking at ~10 min after stimulation and then decreasing, in contrast to PDGF which rises more slowly and continues to increase for at least 3 h. This suggests that PDGF and LPA may possibly be operating through different pathways. LPA has been reported to have actions in fibroblasts besides rho-GTP activation, such as activation of the stimulatory G protein of phospholipase C and the inhibitory G protein of adenylate cyclase (van Corven et al., 1989), release of intracellular Ca^{2+} stores (van Corven et al., 1989; Jalink et al., 1990), and stimulation of arachidonic acid release (van Corven et al., 1989). All of these reported effects of LPA occur at concentrations 100-fold higher than the concentration that induces β-actin mRNA localization (200 ng/ml) with the exception of calcium release. Although an increase of intracellular calcium is reported at similar LPA concentrations that induce localization of β-actin mRNA, the increases occur within seconds after treatment and they decay within 2–3 min (Jalink et al., 1990). In addition, treatment of CEFs with varying concentrations of calcium ionophore A23187 had no effect or caused an inhibition of β-actin mRNA localization (data not shown).

Growth factor signaling has been shown to be influenced by protein kinase activity (Cook and McCormick, 1993; Wu et al., 1993), an observation which illustrates the considerable cross-talk that occurs between cellular signaling pathways. Some of the effects of the cAMP and PKA pathways have been shown to be opposite of that of growth factors whose receptors are tyrosine kinases, such as the effect of cAMP on insulin-stimulated glycogen and triacylglycerol formation (Cohen, 1992) and on PDGF-induced mitogen-activated protein kinase (Graves et al., 1993). In this study we found that activation of both kinases A and C (by forskolin and TPA respectively) inhibited the relocalization of β-actin mRNA in serum-stimulated cells and caused delocalization of mRNA in cells grown in normal growth conditions. The inhibitory effect of these kinases on other PDGF-induced events is downstream of the receptor tyrosine kinase (Heldin et al., 1989; Graves et al., 1993), however beyond this the targets are not known. Activation of ras by PDGF is effected through several intermediate proteins (Lowenstein et al., 1992; Li et al., 1994; Chardin et al., 1993). It is possible that one or more of these receptor-associated molecules is a target for these kinases.

TPA and forskolin have been reported to induce changes in the actin cytoskeleton (Sobue et al., 1988; Lomri and Marie, 1990; Goldman and Abramson, 1990; Perrin et al., 1992). The similarity in the responsiveness of both the mRNA and actin filaments raises the possibility that alterations of phosphorylation delocalize β-actin mRNA by depolymerizing actin filaments. However phalloidin staining of forskolin or TPA-treated cells showed no change in the actin cytoskeleton during the time-course which induced delocalization of β-actin mRNA (not shown). In addition, forskolin did not change the partitioning of β-actin mRNA between detergent soluble and cytoskeletal fractions, with the majority of the mRNA remaining associated with the cytoskeleton. Depolymerization of actin filaments by cytochalasin D results in both delocalization of β-actin mRNA (Sundell and Singer, 1990) and the release of most poly (A) mRNA from the cytoskeleton (Ornelles et al., 1986; Taneja et al., 1992). Therefore forskolin delocalized mRNA is not freely diffusible or released from the cytoskeleton.

Induction of tyrosine kinase activity by PDGF or induction of other kinases may lead to phosphorylation of a protein(s) required for the maintenance of localization. Specific targets of protein kinases could either be motor molecules which move the mRNA or cytoskeletal proteins which anchor the mRNA once it reaches the lamella. Several RNA binding proteins have been shown to be regulated by phosphorylation (see Hershey, 1989). In chick fibroblasts the proximal 3' untranslated region of the β-actin mRNA contains a cis-acting localization sequence (Kislauskis et al., 1993), which supports the idea that specific protein(s) bind to this region and mediate peripheral localization by interacting with the cytoskeleton. Results presented here suggest that these protein(s) may be phosphoproteins. This will aid in the purification, identification, and characterization of the role of these proteins in mRNA sorting.

The modulation of mRNA sorting by growth factors has implications in a wide variety of cellular processes. These include the establishment of both short and long range morphological and physiological effects. The polarity of cells could be effected by asymmetric signals impinging on the cell surface which result in a concomitant asymmetry of protein synthesis. A gradual reorientation of protein synthesis, a mechanism of protein sorting, would change the cells to a stable polarized phenotype. Additionally, cells capable of motility could use the same signals to transiently change direction of migration by supply of specific proteins to the

leading edge without the need for new transcription. Ultimately, the physiological role of spatially directed protein synthesis may be an important component of these processes.

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