c-Src Regulates the Simultaneous Rearrangement of Actin Cytoskeleton, p190RhoGAP, and p120RasGAP following Epidermal Growth Factor Stimulation

Jin-Hong Chang,* Satinder Gill,* Jeffrey Settleman,‡ and Sarah J. Parsons*

*Department of Microbiology and Cancer Center, University of Virginia Health Sciences Center, Charlottesville, Virginia 22908; and ‡Massachusetts General Hospital Cancer Center, Charlestown, Massachusetts 02129

Abstract. Analysis of C3H10T1/2 murine fibroblasts overexpressing wild type and dominant negative variants of c-Src has demonstrated a requirement for c-Src in EGF-induced mitogenesis. Correlating with the ability of c-Src variants to potentiate or inhibit EGF-dependent DNA synthesis is the phosphotyrosine content of multiple cellular proteins, including p190-RhoGAP, a protein thought to regulate growth factor–induced actin cytoskeleton remodeling by modulating the activity of the small GTP binding protein, Rho. Because the in vivo phosphotyrosine content of p190 varies with the level of active c-Src and not with EGF treatment, p190 is considered to be a preferred substrate of c-Src. To determine whether tyrosyl phosphorylation of p190 (by c-Src) could influence EGF-dependent actin remodeling, we used conventional and confocal immunofluorescence microscopy to examine the intracellular distribution of p190, actin, and p120RasGAP in EGF-stimulated or unstimulated 10T1/2 Neo control cells and cells that stably overexpress wild-type (K^+) or kinase-defective (K^-) c-Src. We found that in all cell lines, EGF induced a rapid and transient condensation of p190 and RasGAP into cytoplasmic, arclike structures. However, in K^+ cells the rate of appearance and number of cells exhibiting arcs increased when compared with control cells. Conversely, K^- cells exhibited delayed arc formation and a reduction in number of cells forming arcs. EGF-induced actin stress fiber disassembly and reassembly occurred with the same kinetics and frequency as did p190 and RasGAP rearrangements in all three cell lines. These results, together with the documented Rho-GAP activity intrinsic to p190 and the ability of Rho to modulate actin stress fiber formation, suggest that c-Src regulates EGF-dependent actin cytoskeleton reorganization through phosphorylation of p190.

C-SRC is a ubiquitous, nonreceptor tyrosine kinase that localizes intracellularly to the cytoplasmic face of the plasma membrane and to poorly defined structures surrounding the nucleus (12, 35). Functionally, it is involved in a variety of biological processes which are associated with cytoskeletal reorganization, including malignant transformation (43), neuronal differentiation (28, 37), bone remodeling (62), and regulated secretion (17, 42). In addition, a requirement for c-Src and its family members in EGF-, PDGF-, and colony-stimulating factor I–dependent mitogenesis has been demonstrated by overexpression and microinjection studies (56, 65, 70). For example, stable overexpression of wild-type (*wt*[1] or K^+) c-Src in C3H10T1/2 murine fibroblasts enhances EGF-induced mitogenesis (33), while overexpression of the dominant negative kinase defective (K^-) c-Src inhibits the normal response (70). Effects of K^+ and K^- overexpression on DNA synthesis correlate with the level of tyrosyl phosphorylation on a number of cellular proteins and permit their classification as preferred substrates of the EGF receptor or c-Src (69).

Considerable evidence indicates that tyrosine kinases of both the receptor and nonreceptor types influence actin cytoskeleton dynamics. PDGF stimulation induces cytoskeletal rearrangements that result in the formation of perinuclear, ring-shaped membrane ruffles in human foreskin fibroblasts (23). Overexpression of kinase-defective PDGF receptors prevents ruffling, indicating that the formation of such structures is dependent on receptor tyrosine kinase activity. Similar observations were made when the receptor for hemapoietic stem cell factor (c-kit) was expressed and activated in endothelial cells (3), and when human A431 carcinoma cells were treated with EGF (55). den Hartigh et al. (13) also demonstrated binding of the EGF receptor to actin itself. These findings suggest that receptor tyrosine kinases play an important role in ac-

Address all correspondence to Dr. Sarah J. Parsons, Department of Microbiology and Cancer Center, University of Virginia Health Sciences Center, Box 441, Charlottesville, VA 22908. Tel.: (804) 924-2352. Fax: (804) 982-0689.

1. *Abbreviations used in this paper*: K^+, C3H10T1/2 fibroblasts overexpressing wild-type c-Src; K^-, 10T1/2 fibroblasts overexpressing kinase defective c-Src; *wt*, wild-type.

tin-based cytoskeletal reorganization after growth factor stimulation.

Nonreceptor tyrosine kinases are also implicated in actin cytoskeletal dynamics. Both the myristoylated, nontransforming c-Abl protein and the transforming p210Bcr/Abl protein associate with F-actin stress fibers in situ (39, 66), and the COOH terminus of c-Abl can mediate bundling of F-actin filaments in vitro (67). Introduction of v-Src into fibroblasts results in a dramatic disassembly of the actin cytoskeleton, formation of short actin filaments, weak association of actin filaments with the membrane, and reduction in adherence (18). The constitutively altered morphology which results from transformation by v-Src contrasts sharply with the transient actin reorganization after growth factor stimulation of normal fibroblasts. Nevertheless, several lines of evidence suggest that the normal homologue of v-Src, c-Src, plays a role in regulating actin rearrangements in normal cells. These include the documented effects of v-Src on the actin cytoskeleton, the demonstrated requirement for c-Src in growth factor–mediated mitogenesis, and the identification of several cytoskeleton-associated proteins as c-Src substrates (below). However, the mechanism by which c-Src may participate in mitogen-induced cytoskeletal rearrangements is far from clear.

c-Src substrates that appear to be involved in actin network dynamics include cortactin (35, 71), a filamentous actin-binding protein enriched in the cell cortex (72), and p190, a tyrosyl-phosphorylated protein that coprecipitates with the GTPase-activating protein of p21Ras (p120RasGAP) (6, 10, 15). Purification of p190 and molecular cloning of its cognate cDNA revealed sequences of a multifunctional protein that include an NH_2-terminal Ras-like GTPase domain, an uncharacterized central region, and a COOH-terminal GAP domain for the small GTP-binding proteins, Rac and Rho (60).

Recent evidence indicates that the Rac/Rho family of small GTP-binding proteins is involved in reorganization of actin cytoskeletal structures after mitogenic stimulation (30, 32). FCS and lysophosphatidic acid induce rapid stress fiber formation (within 5 min) but little or no ruffling in Swiss 3T3 cells, while PDGF and EGF are slow to induce the formation of stress fibers (~30 min) but stimulate rapid membrane ruffling (within 10 min) (51). In these cells, stress fiber formation is stimulated by microinjection of Rho, and blocked by C3 exotransferase, which selectively inhibits Rho. Microinjection of Rho does not appear to induce membrane ruffling. Rather, using constitutively activated or dominant negative forms of Rac, Ridley et al. (53) demonstrated that Rac GTPases regulate membrane ruffling. Thus, they conclude that Rho regulates actin stress fiber and focal adhesion formation in Swiss 3T3 cells, while Rac regulates membrane ruffling at the plasma membrane.

Functional analysis of the COOH-terminal region of p190 shows that it contains a GAP activity that down-regulates Rac and Rho in vitro (59). In addition, Ridley et al. (54) demonstrated that microinjection of the p190GAP region into Swiss 3T3 cells blocks serum-induced stress fiber formation, but does not block PDGF-induced membrane ruffling. These results suggest that the GAP domain of p190 preferentially regulates stress fiber formation in response to growth factor stimulation, by regulating Rho. Furthermore, p190 also appears to function in conjunction with RasGAP, since overexpression of the NH_2 terminus of RasGAP results in its constitutive association with p190RhoGAP, as well as induction of membrane ruffling, disruption of the actin cytoskeleton, and a decrease in fibronectin binding and cell adhesion (38). Taken together, these findings indicate that p190 may mediate mitogen-dependent actin cytoskeletal reorganization by regulating Rho activity.

Previous studies demonstrated that p190 is a preferred substrate of c-Src in 10T1/2 cells, i.e., it is highly tyrosyl phosphorylated in cells overexpressing *wt* c-Src (K^+ cells) and poorly phosphorylated in cells overexpressing kinase defective c-Src (K^- cells) (10). Furthermore, the level of p190 tyrosyl phosphorylation is not affected by EGF stimulation. To determine whether tyrosyl phosphorylation of p190 (by c-Src) could affect the ability of p190 to regulate actin stress fiber formation, we used confocal and conventional immunofluorescence microscopy to study changes in the intracellular distribution of p190, RasGAP, and F-actin after EGF stimulation in K^+, K^-, and Neo control cells. We found that in quiescent cells of all three types, p190 and RasGAP colocalized to the cytoplasm in a heterogenous pattern. Within seconds to minutes after EGF stimulation, p190 and RasGAP cocondensed into arclike structures that radiated away from the nucleus in a concentric fashion. Arcs were transient in all cell lines, but they were detected earlier and for a longer period of time in K^+ cells (which contain highly tyrosyl-phosphorylated p190) than in Neo control cells. The opposite was seen in K^- cells (which contain little or no tyrosyl-phosphorylated p190), where arc formation was delayed and occurred over a shorter period of time. Significantly, EGF-induced actin stress fiber dissolution coincided with p190/RasGAP arc formation in all three cell lines. Furthermore, the extent of actin rearrangement was found to be more pronounced in K^+ cells and less pronounced in K^- cells compared with controls. p190 arc structures appeared to delimit the region of highest actin dissolution, consistent with the idea that p190 regulates actin stress fiber dynamics. Taken together, these findings support a model in which p190 mediates actin cytoskeleton changes upon reception of at least two signals, one from the growth factor receptor, and another from c-Src.

Materials and Methods

Polyclonal and Monoclonal Antibody Production

Rabbit Anti-RasGAP Polyclonal Antibodies. A bacterial *trp*E fusion protein containing the entire RasGAP molecule except the six NH_2-terminal amino acids was expressed in *Escherichia coli* from a pATH1 plasmid (31), generated by insertion of the NaeI fragment of bovine RasGAP cDNA (68) into the SmaI site of the vector. *trp*E-RasGAP was purified from bacterial cell lysates by SDS-PAGE and elution from excised gel pieces in PBS (136 mM NaCl, 2.6 mM KCl, 10 mM Na_2HPO_4, 1.7 mM KH_2PO_4, pH 7.2). Soluble, purified protein was emulsified with Freund's adjuvant and injected into New Zealand White rabbits according to the immunization protocol described by Ely et al. (16). Antibodies were purified from immune sera first by protein A chromatography as described by Harlow and Lane (25) and Zola (73) and subsequently by GST-RasGAP-SH2-SH3-SH2 Sepharose affinity chromatography according to the procedure of Smith and Johnson (61).

Murine Anti-RasGAP Monoclonal Antibodies. *Trp*E-RasGAP was also used to generate mAbs. Immunization, fusion, and culturing procedures were carried out as described by Chang et al. (11) and were performed by the University of Virginia Lymphocyte Culture Facility (Charlottesville, VA). 26 hybridoma supernatants were generated that specifically immunoblotted and immunoprecipitated p120RasGAP from lysates of IV5 cells, a v-Src transformed C3H10T1/2 murine fibroblast cell line (33). Epitope mapping using bacterially expressed domains of RasGAP revealed three binding specificities, the SH3 domain, the calcium binding domain, and combinatorial sequences derived from noncontiguous regions of the molecule (Chang, J.-H., S. Gill, and S. J. Parsons, unpublished results; 8). mAb 6F2, which was used in this study, bound the SH3 domain. Selected mAbs (including 6F2) were also found to be cross-reactive with RasGAP from other species, including human, monkey, chicken, and mouse. mAbs were purified from either culture supernatant or ascites by protein G chromatography (Pharmacia LKB Biotechnology Inc., Piscataway, NJ) according to manufacturer's instructions.

Anti-p190RhoGAP Polyclonal and Monoclonal Antibodies. A *trp*E bacterial fusion protein containing full-length p190 was generated by inserting a PCR product of clone 391 (60) into the pATH11 vector. The PCR product lacked both 5′ and 3′ noncoding sequences of the original clone and was engineered to contain EcoRI restriction sites at either end to facilitate cloning. *trp*E-p190 fusion protein was purified as described above and used for immunizing rabbits. Polyclonal rabbit antibodies were purified from immune sera by protein A and subsequently by *trp*E-p190 affinity chromatography. *trp*E-reactive antibodies were removed by adherence to *trp*E matrixes. For generation of p190 mAbs, mice were immunized with a mixture of *trp*E-p190 and gel-purified p190 isolated from p120RasGAP immunoprecipitates derived from IV5 cells. Hybridoma supernatants were screened for their ability to immunoblot and immunoprecipitate p190 from IV5 cells. Three of these hybridomas (3D4, 8C10, and 3F9) were selected for these studies.

Cells

The derivation and characterization of the stable transfectants of C3H-10T1/2 murine fibroblasts used in this study have been described previously (10, 33, 70). Briefly, clones were selected for neomycin resistance after transfection of pSV2neo alone or in combination with a plasmid encoding K^+ or K^- c-Src. Clone 5Hd47 expresses wild-type (*wt* or K^+) chicken c-Src at a level 15–30-fold above endogenous. Clone 430-62 expresses nearly identical levels as 5Hd47, but harbors a K^- c-Src, which contains an A430V mutation in the kinase domain, rendering the protein enzymatically inactive. A mixture of clones exhibiting neomycin resistance alone were used as controls. Both K^+ and K^- c-Src proteins were found to partition appropriately to the insoluble fraction when cells were subjected to hypotonic lysis and differential centrifugation. K^+ c-Src from 5Hd47 cells was ascertained to be enzymatically active and to exhibit a specific kinase activity similar to endogenous c-Src. K^- c-Src was verified to be catalytically inactive. All cell lines maintained normal morphology and were similar to one another with respect to growth rate, saturation density, etc., in serum, but were distinctly different in their ability to synthesize DNA when stimulated with individual growth factors, such as EGF (33, 70). 5Hd47 and 430-62 were representative of multiple clones overexpressing K^+ or K^- c-Src, respectively, with regard to their EGF responsiveness. The cells were maintained in DME containing 10% (vol/vol) FCS, penicillin/streptomycin, and G418 (400 μg/ml; GIBCO BRL, Gaithersburg, MD).

Immunoprecipitation and Western Immunoblotting

Mitogen stimulation of quiescent cells, preparation of cellular lysates, immunoprecipitations, and Western immunoblotting were carried out as described by Wilson et al. (70) and Chang et al. (10). Briefly, cells were grown to confluence, deprived of serum for 24 h, and stimulated with 100 ng EGF for various times. Lysates were prepared by addition of ice-cold RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.25% sodium deoxycholate, 0.1% SDS, 1% NP-40, 1 mM sodium orthovanadate, 1 mM PMSF, 50 μg/ml leupeptin, and 0.5% aprotinin) to the monolayer and centrifugation of the extract for 30 min (100,000 *g*) at 4°C. Protein concentrations of the supernatants were determined by the bicinchoninic acid assay (Pierce Chemical Co., Rockford, IL). For immunoblotting, 100 μg protein extract was resolved by electrophoresis through 6% polyacrylamide gels, transferred to Immobilon membranes (Millipore Corp., Bedford, MA), and incubated with 1 μg/ml purified rabbit or mouse α-p190 or α-RasGAP Ig in blotting buffer. Phosphotyrosine-containing proteins were detected with mAb 4G10 (Upstate Biotechnology, Inc., Lake Placid, NY). Binding of rabbit Ig to cellular proteins was revealed by ^{125}I-protein A, while binding of mouse mAbs was detected by incubation with 1 μCi/ml ^{125}I-goat anti-mouse IgG (100 μCi/ml). Both radioactive compounds were obtained from New England Nuclear (Boston, MA).

For immunoprecipitations, 500 μg lysate protein was incubated with 1–10 μg purified α-p190 or α-RasGAP Ig at 4°C for 1 h. Immunocomplexes were recovered by incubation for 30 min with 100 μl of a 10% suspension of protein A-Sepharose. When added to mouse mAbs, protein A-Sepharose was preincubated with 10 μg affinity-purified, rabbit α-mouse IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Immunocomplexes were then washed three times with RIPA buffer in preparation for immunoblotting.

Immunofluorescence

Cells were seeded onto glass coverslips, grown to near confluency, deprived of FCS for 24 h, and stimulated with 100 ng/ml EGF for various lengths of time (0–24 h) in a tissue culture incubator maintained at 37°C. All subsequent manipulations were carried out at room temperature. Primary (1°) antibodies were affinity purified, and purified secondary (2°) and tertiary (3°) antibodies were adsorbed to eliminate cross-reactivity with mouse tissues. All antibodies were used at a concentration of 10 μg/ml. For actin localization, cells were fixed in 3% paraformaldehyde for 20 min, permeabilized with 0.4% Triton X-100 for 4 min, and incubated with FITC-labeled Phalloidin (Molecular Probes Inc., Eugene, OR) at a concentration of 5 U/ml for 40 min. Coverslips were washed three times with PBS between each treatment or antibody incubation. For localization of p190 or RasGAP alone (i.e., single antibody labeling experiments), cells were fixed and permeabilized as described above, and incubated sequentially with 1° rabbit polyclonal antibody or mouse mAb, 2° goat anti-rabbit or goat anti-mouse IgG, 8% donkey serum (to block nonspecific binding of the 3° antibody), and 3° FITC-conjugated donkey anti-goat IgG for 40 min each before the final wash. For double labeling, combinations of purified rabbit polyclonal or mouse mAb specific for either p190 or RasGAP were used as 1° antibodies, and appropriate 2° and 3° antibodies were applied, as indicated in the figure legends. All 2° and 3° antibodies were obtained from Jackson ImmunoResearch Laboratories, Inc. Coverslips were mounted on microscope slides, and cells were viewed on a confocal microscope (MRC600; Bio-Rad Laboratories, Richmond, CA) or a fluorescent microscope (Orthoplan; E. Leitz, Inc., Rockleigh, NJ). Confocal images were processed by the Adobe Photoshop software program (Adobe Systems, Inc., Mountain View, CA).

Results

P190: a Preferred Substrate for c-Src

We previously demonstrated that the phosphotyrosine content of p190 that coprecipitated with RasGAP was elevated in cells overexpressing K^+ c-Src and diminished in cells overexpressing the dominant negative (K^-) form of c-Src when compared with control cells (10). It was postulated that such differences might affect the function or subcellular distribution of p190. To determine if total p190 (RasGAP-associated and nonassociated) from the three cell lines exhibited similar differences in levels of phosphotyrosine as did RasGAP-associated p190 alone, we quantitatively immunoprecipitated p190 from whole-cell extracts of K^+, K^-, and control cells, and divided the immunoprecipitate into two parts. One-half was blotted with phosphotyrosine antibody, and the other with p190 antibody. Fig. 1 *A* shows that overexpression of K^+ c-Src resulted in a three- to fourfold increase in p190 tyrosyl phosphorylation (lanes *7–9*) as compared with Neo control cells (lanes *1–3*), without affecting p190 protein levels (Fig. 1 *B*, compare lanes *7–9* of K^+ cells with lanes *1–3* of control cells). Overexpression of K^- c-Src resulted in a striking reduction in p190 tyrosyl phosphorylation (Fig. 1 *A*,

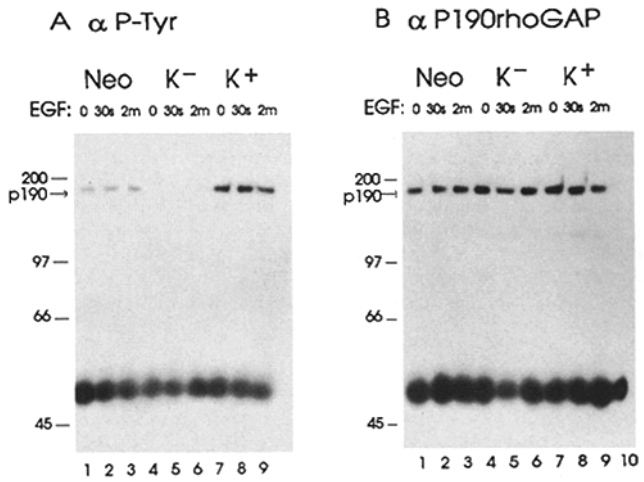

Figure 1. p190 is a c-Src substrate. Immunoprecipitates were prepared from 500 μg lysate protein derived from Neo control, K^+, or K^- cells (before or after stimulation with 100 ng/ml EGF) using rabbit α-p190 (lanes *1–9*) or normal rabbit serum (lane *10*) as described in Materials and Methods. Each precipitate was divided into two parts. One half (*A*) was analyzed by immunoblotting with α-p-Tyr antibody (4G10), and the other half (*B*) was immunoblotted with rabbit α-p190. (*Arrow*) Position of p190.

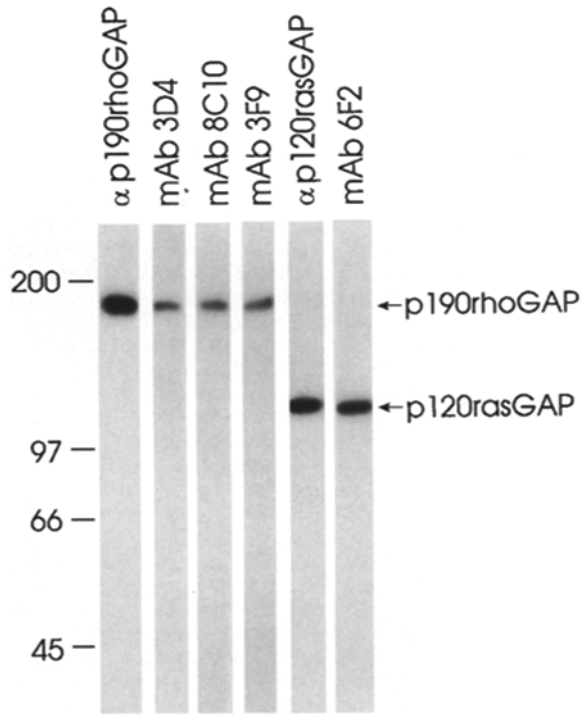

Figure 2. Specificity of p190 and RasGAP polyclonal and monoclonal antibodies used in this study. 100 μg K^+ cell lysate protein was analyzed by Western immunoblotting with polyclonal or mAbs specific for p190 (rabbit polyclonal α-p190RhoGAP and mAbs 3D4, 8C10, and 3F9) or RasGAP (rabbit polyclonal α-p120RasGAP and mAb 6F2). Antibodies were affinity purified as described in Materials and Methods, concentrated to 0.4–1.0 mg/ml, and used at a dilution of 1:1,000 in blocking solution. Rabbit IgG bound to cellular protein was detected with ^{125}I-labeled protein A, and mouse IgG was detected with ^{125}I-labeled goat anti–mouse IgG. (*Arrows*) Positions of p190 and RasGAP. Molecular mass standards are denoted as kD.

lanes *4–6*), again, without affecting the level of p190 protein (*B*, lanes *4–6*). Thus, total p190 exhibited the same relative differences in levels of tyrosyl phosphorylation in the various cell lines as did RasGAP-associated p190. Furthermore, the level of p190 tyrosyl phosphorylation was not altered by EGF stimulation within the time frame most EGF receptor substrates become phosphorylated in 10T1/2 cells (10, 69). Taken together, these results indicate that p190 is a preferred substrate of c-Src vs. the EGF receptor, and that overexpression of c-Src does not affect p190 expression levels. Previous work also had demonstrated that neither RasGAP nor EGF receptor protein levels are altered by c-Src overexpression (10, 33).

Intracellular Localization of p190 after EGF Stimulation: formation of Concentric Arcs in the Cytoplasm

To investigate the intracellular distribution of p190 before and after EGF stimulation, we generated rabbit polyclonal and mouse mAbs to p190RhoGAP and p120RasGAP as described in Materials and Methods. To verify the specificity of the antibodies and their suitability for immunofluorescence microscopy, the antibodies were tested by direct Western immunoblot analysis of C3H10T1/2 whole-cell lysates. Fig. 2 shows that both the rabbit polyclonal and mouse mAbs (3D4, 8C10, and 3G12) raised to p190RhoGAP reacted with a single band of 190 kD. Similarly, both the p120RasGAP polyclonal antibody and mAb 6F2 reacted specifically with a single band of 120 kD. Thus, the antibodies did not exhibit detectable cross-reactivities for other cellular proteins and were considered suitable reagents for immunofluorescence.

To determine the subcellular localization of p190, Neo control cells and cells overexpressing K^+ or K^- c-Src were grown on glass coverslips, deprived of serum for 24 h, stimulated for varying lengths of time with EGF (0–30 min), immunostained with rabbit polyclonal p190 antibody, and examined by conventional immunofluorescence microscopy. Fig. 3, *A* and *B*, shows that in the absence of EGF or 30 s after stimulation of Neo control cells, p190 localized predominantly to the perinuclear/nuclear region, exhibiting diffuse, punctate, and network staining, as well as some decoration of actin stress fibers. Within 2 min of EGF stimulation, however, p190 condensed in the cytoplasm to form wavelike, concentric arcs radiating away from the nucleus (Fig. 3 *C*, *arrows*). Arcs were transient, and staining returned to the more heterogeneous pattern seen at quiescence ~5 min after stimulation (*D* and *E*). Similar, but not as well-defined arcs were detected in response to PDGF stimulation of Neo cells (data not shown).

Regulation of the Timing of p190 Arc Formation by c-Src

To investigate the effects of c-Src on the intracellular localization of p190, cells overexpressing K^+ and K^- c-Src also were stained with anti-p190 and examined by immunofluorescence after EGF stimulation. Fig. 3, *G* and *M*, shows that in quiescence, the localization of p190 in cells overexpressing K^+ and K^- c-Src was similar to that seen in

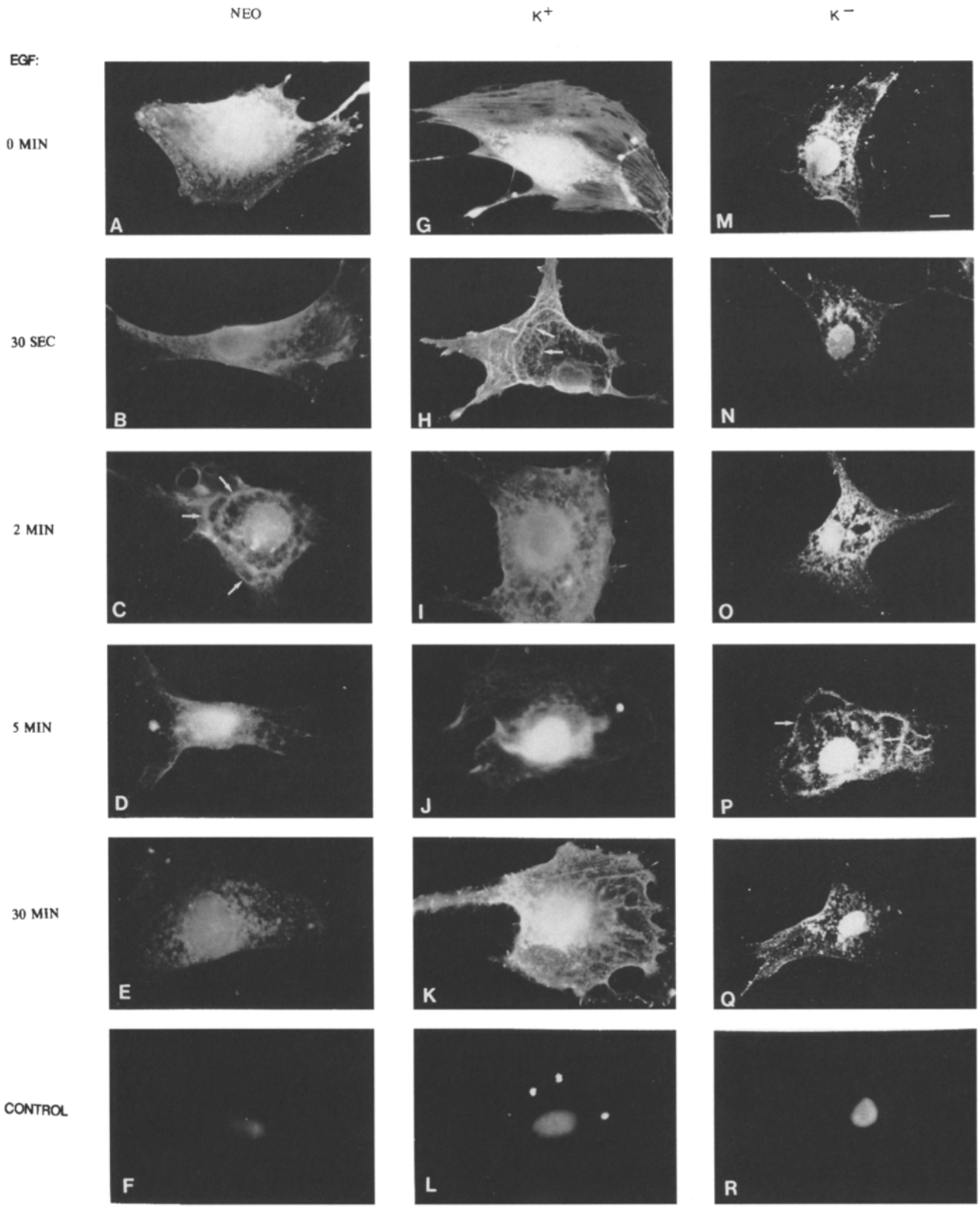

Figure 3. Regulation of p190 redistribution by EGF and c-Src. Neo control, K^+, and K^- cells were grown on coverslips, starved of serum, and either mock-stimulated or stimulated with 100 ng/ml EGF for the indicated times before immunostaining with affinity-purified rabbit polyclonal α-p190 (*A–E*, *G–K*, and *M–Q*) or purified IgG from normal rabbit sera (*F*, *L*, and *R*) as described in Materials and Methods. Samples (*A–L*) were developed with FITC-conjugated donkey α-goat IgG for visualization through a Leitz microscope equipped with epi-illumination, and (*M–R*) were incubated with Cy-3–conjugated donkey α-goat IgG for visualization through a confocal microscope. Background staining in *F*, *L*, and *R* was similar to that seen when 1° antibody was omitted or α-p190 was preblocked with purified immunogen. Nuclear staining was variable and may or may not be physiologically relevant. EGF induced the formation of concentric arcs containing p190 (*C*, *H*, and *P*). Overexpression of *wt* c-Src accelerated the appearance of these arcs (*H*), while overexpression of kinase-defective c-Src delayed their formation (*P*). Bar, 10 μm.

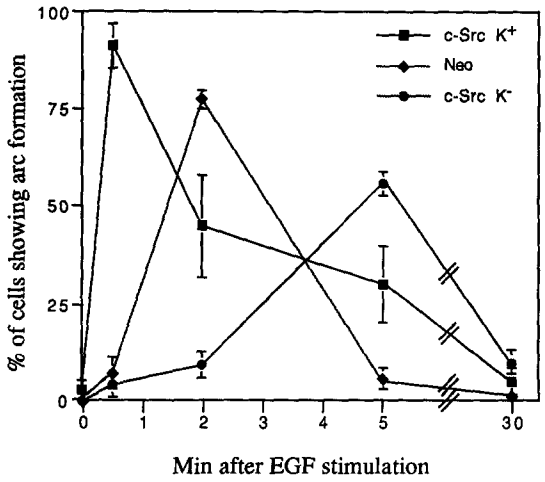

Figure 4. Percentage of Neo control, K^+, and K^- cells exhibiting concentric arcs after EGF stimulation. Cells were stimulated with 100 ng/ml EGF for the indicated times and immunostained with rabbit α-p190 as in Fig. 3. 150–200 cells at each time point for each cell type were scored for the presence of concentric arcs or wavelike structures. Results are depicted as the average values obtained from two independent experiments ± SEM.

Neo control cells. Upon EGF stimulation of K^+ cells, arcs formed consistently more rapidly, i.e., within 30 s of EGF stimulation (*H, arrows*), while in K^- cells, arc formation was delayed, first appearing around 5 min of EGF stimulation (*P, arrows*). In both K^+ and K^- cells, arcs were transient. Examination of the three cell lines at intervals of every 2–4 h for up to 24 h of continual EGF treatment revealed no additional alterations in p190 distribution (data not shown).

To quantitate the number of cells in a population displaying concentric arcs, 150–200 cells at each time point (0, 30 s, 2 min, 5 min, and 30 min after EGF addition) for each cell type were scored for the presence of arcs in two independent experiments. Fig. 4 demonstrates that the largest number of cells displaying clearly demarcated concentric arcs occurred at 30 s in K^+ cells, 2 min in Neo control cells, and 5 min in K^- cells. Overexpression of K^+ c-Src not only accelerated arc formation, but also appeared to extend the time frame in which it occurred (from ~30 s to >5 min for K^+ cells vs. from ~2 min to ~5 min for Neo cells), resulting either in greater numbers of cells undergoing the rearrangement or arcs of longer duration. In contrast, fewer K^- cells formed concentric arcs. These differences are detailed in Table I. While the total number of K^+ and Neo cells exhibiting concentric arcs varied among experiments, the number of arc-containing K^- cells was always proportionally less than the number of arc-containing K^+ and Neo cells (~40 and ~75% of K^+ and Neo cells, respectively).

Regulation of Actin Redistribution by EGF and c-Src

The small GTP-binding proteins, Rac and Rho, have been implicated in actin cytoskeletal rearrangement, as microinjection of these proteins affects membrane ruffling and stress fiber/focal adhesion formation (51, 53). The COOH-terminal domain of p190 also has been demonstrated to contain a GAP activity for Rho/Rac (59), and microinjection of p190GAP has been shown to affect actin cytoskeletal rearrangements (54). To investigate whether actin reorganization occurs after EGF stimulation in 10T1/2 cells, and whether differential tyrosyl phosphorylation of p190 might correlate with such rearrangements, Neo, K^+, and K^- cells were grown to near confluency on glass coverslips, starved of serum for 24 h, stimulated with EGF, and labeled with phalloidin to visualize actin filaments. Fig. 5, *A*, *F*, and *K*, shows that in all three cell lines, actin filaments were bundled into macrofilaments and stress fibers during quiescence. Upon EGF stimulation, however, a striking reorganization of F-actin occurred, including the dissolution of stress fibers and the appearance of short, fine filamentous structures (*C*, *G*, and *N*). The peak time at which actin reorganization occurred differed with each cell line, and interestingly, coincided with the peak times

Table I. Quantification of p190 Arc Formation and Actin Rearrangement

Cell lines	Minutes after EGF stimulation	Percentage of cells exhibiting p190 concentric arcs		Percentage of cells undergoing actin rearrangement	
		Experiment 1	Experiment 2	Experiment 3	Experiment 4
c-Src K^+	0	0	5	0	0
	0.5	97	86	97	79
	2	58	32	83	56
	5	40	20	30	31
	30	11	0	0	23
Neo	0	0	1	0	0
	0.5	4	10	12	15
	2	80	75	97	73
	5	4	7	6	10
	30	2	1	2	0
c-Src K^-	0	0	0	0	0
	0.5	7	1	2	6
	2	6	12	3	6
	5	53	59	50	48
	30	0	15	25	0

K^+, Neo control, and K^- cells were treated with EGF for the indicated times, stained with either p190 polyclonal antibody (Experiments 1 and 2) or phalloidin (Experiments 3 and 4) as described for Figs. 3 and 6, respectively, and examined by confocal or conventional fluorescence microscopy. The percentage of cells exhibiting p190 arc formation or actin stress fiber disassembly was determined by scoring the number of positive cells in a total of 150–200 for each cell line at each time point.

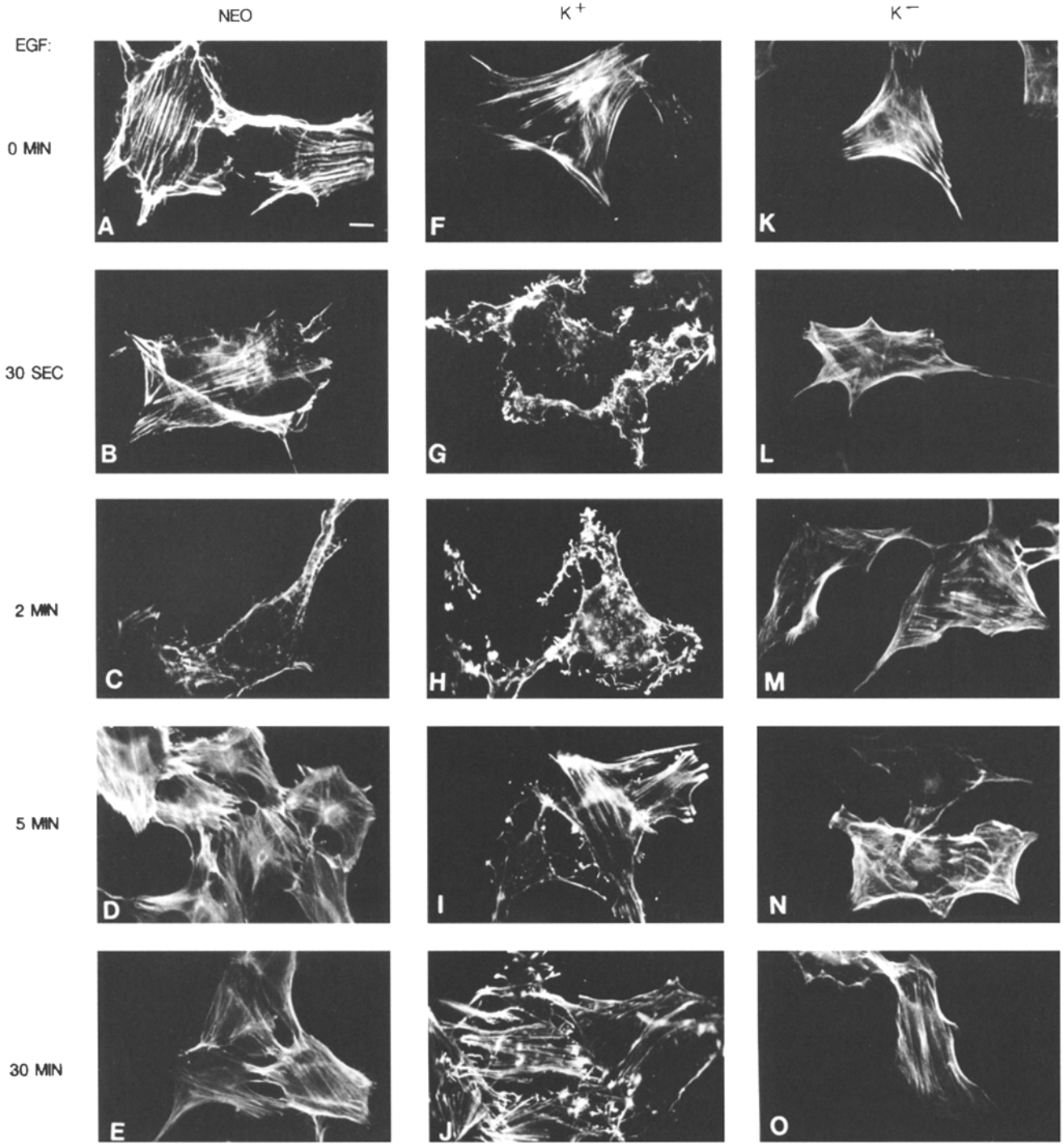

Figure 5. Actin cytoskeletal redistribution after EGF stimulation. Neo control, K^+, and K^- cells were starved of serum for 24 h, stimulated with 100 ng/ml EGF for the indicated times, and incubated with fluorescein-conjugated phalloidin to stain actin filaments. Images were recorded by confocal microscopy. Actin rearrangement and dissolution occurred with the same appropriate kinetics as did p190 arc formation in the three cell types. Bar, 10 μm.

of p190 arc formation (i.e., 30 s for K^+ cells [*G*], 2 min for Neo control cells [*C*], and 5 min for K^- cells [*N*]) (also see Table I for quantitation). As with p190 arc formation, actin dissolution was transient, and stress fibers reformed within 5 min of stimulation for Neo control cells (*D*). However, while the bulk of disorganized F-actin in K^+ cells reformed stress fibers within 30 min of EGF stimulation (*J*), reformation was less complete and slower than in Neo cells, indicating that overexpression of K^+ c-Src promoted actin disruption. The opposite was observed in K^- cells, where actin cytoskeletal dissolution was delayed and less complete compared with Neo and K^+ cells (*N*), and the appearance of reformed stress fibers at 30 min after stimulation (*O*) was indistinguishable from those at quiescence (*K*). Thus, EGF-dependent actin dissolution was accelerated, prolonged, and more pronounced in the pres-

ence of K^+ c-Src, and delayed and less pronounced in the presence of K^- c-Src. Moreover, quantification revealed that the time after EGF stimulation at which the most extensive actin dissolution occurred in the greatest number of cells coincided with the peak time of formation of p190-containing concentric arcs in the various cell lines (Fig. 6 and Table I). This correlation, together with the known effects of p190RhoGAP on Rho/Rac and Rho/Rac on actin, suggests that tyrosyl phosphorylation of p190 plays a role in regulating the timing and extent of actin dissolution following growth factor stimulation.

Spatial Relationship between p190 Arcs and Actin Filaments after EGF Stimulation

To investigate the spatial relationship between p190 arcs and actin structures induced by EGF, K^+ cells were double-labeled with p190 mAb 3D4 and phalloidin before and after EGF stimulation. Fig. 7, *A* and *D*, shows that at quiescence, p190 did not colocalize with actin stress fibers or macrofilaments. Within 30 s of EGF stimulation, however, stress fiber dissolution occurred simultaneously with the formation of p190 arcs (*B* and *E*). Actin underwent the greatest depolymerization in the region surrounding the nucleus, and only the barest outlines of phalloidin-positive filaments could be discerned, due to the high degree of actin disassembly. However, these outlines appeared to coincide with the location of p190 arcs (*B* and *E*, *arrows*), suggesting that the arcs may represent a boundary between actin that is partially depolymerized and actin that is monomeric.

To investigate further the idea that p190 could be localizing to the same sites as partially depolymerized actin, we used cytochalasin as an actin-disrupting agent (58), whose ability to induce disassembly of actin could be controlled better than that of EGF. K^+ cells were treated with various concentrations of cytochalasin for 10 min at 37°C, and then immunostained with p190 mAb 3D4 and phalloidin. Fig. 8 *F* shows that when cells were treated with 1 μM cytochalasin, actin stress fibers and macrofilaments were partially disrupted, and some clustering and condensation of F-actin short forms could be seen. At 10 μM, however, cytochalasin induced the formation of actin-containing cytoplasmic structures (Fig. 8 *G*, *arrows*) and p190 formations (Fig. 8 *C*, *arrows*) that mimicked in shape and location the EGF-induced p190 arcs we had observed in previous experiments (Fig. 3, *C*, *H*, and *P*; Fig. 7 *B*). At this concentration of cytochalasin, P190 and partially depolymerized actin were seen to colocalize at many sites throughout the cells. Upon treatment with 30 μM cytochalasin, actin filaments disappeared, and phalloidin highlighted diffuse and globular structures distributed throughout the cell (Fig. 8 *H*). P190 also was seen to distribute throughout the cell, but in a fine network pattern. It did not colocalize with actin (Fig. 8 *D*). These results indicate that actin and p190 are capable of colocalizing when actin undergoes rearrangements and that this colocalization appears to occur at a midstage in actin depolymerization that results in partial dissolution of stress fibers. Thus, the coincidence of p190 arcs with the boundaries between partially depolymerized and monomeric actin in EGF-stimulated cells could represent colocalization of p190 with partially depolymerized actin.

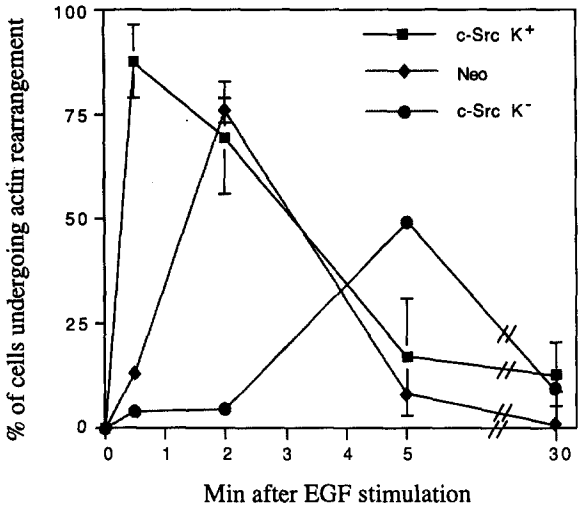

Figure 6. Percentage of Neo control, K^+, and K^- cells exhibiting actin dissolution after EGF stimulation. Cells were stimulated with 100 ng/ml EGF for the indicated lengths of time and stained with phalloidin as in Fig. 5. 150–200 cells at each time point for each cell type were scored for actin dissolution. Results are depicted as the average values obtained from two independent experiments ± SEM.

Colocalization of p190 and RasGAP

Tyrosyl-phosphorylated p190 has been shown to be associated physically with RasGAP in cultured fibroblasts (6, 10, 15, 26, 49, 50). To determine if RasGAP displayed a similar rearrangement as that seen for p190 after EGF stimulation, K^+ cells were double-labeled with p190 and RasGAP antibodies, and the proteins were visualized by confocal immunofluorescence microscopy. Fig. 9, *A* and *G*, shows that in quiescent cells, both p190 and RasGAP localized to the perinuclear region. Within 30 s of EGF stimulation, both proteins formed arclike structures that were superimposable (*B* and *H*, *arrows*). Within 5 min of EGF stimulation p190 and RasGAP returned to the more unorganized pattern seen in quiescent cells (*D* and *J*, respectively). RasGAP also colocalized with p190 in quiescent Neo control and K^- cells and underwent reorganization after EGF stimulation with the same kinetics as p190, mimicking the peak time points of 2 and 5 min for arc formation, respectively (data not shown). Together these findings demonstrate that RasGAP colocalizes with p190 both before and after growth factor stimulation, and is subject to the same temporal regulation by c-Src as is p190. However, RasGAP is not tyrosyl phosphorylated in any of the cell lines used in this study (10), suggesting that its spatial and kinetic distribution is mediated through its association with p190.

Discussion

Signaling Pathways from Growth Factor Receptors to the Actin Cytoskeleton

Restructuring of the actin-based cytoskeletal network is an early event that occurs in response to a variety of extracellular and intercellular signals (7, 9, 23, 51, 53, 55). In many cell types, growth factor–induced actin restructuring correlates with the breakdown of stress fibers, rearrange-

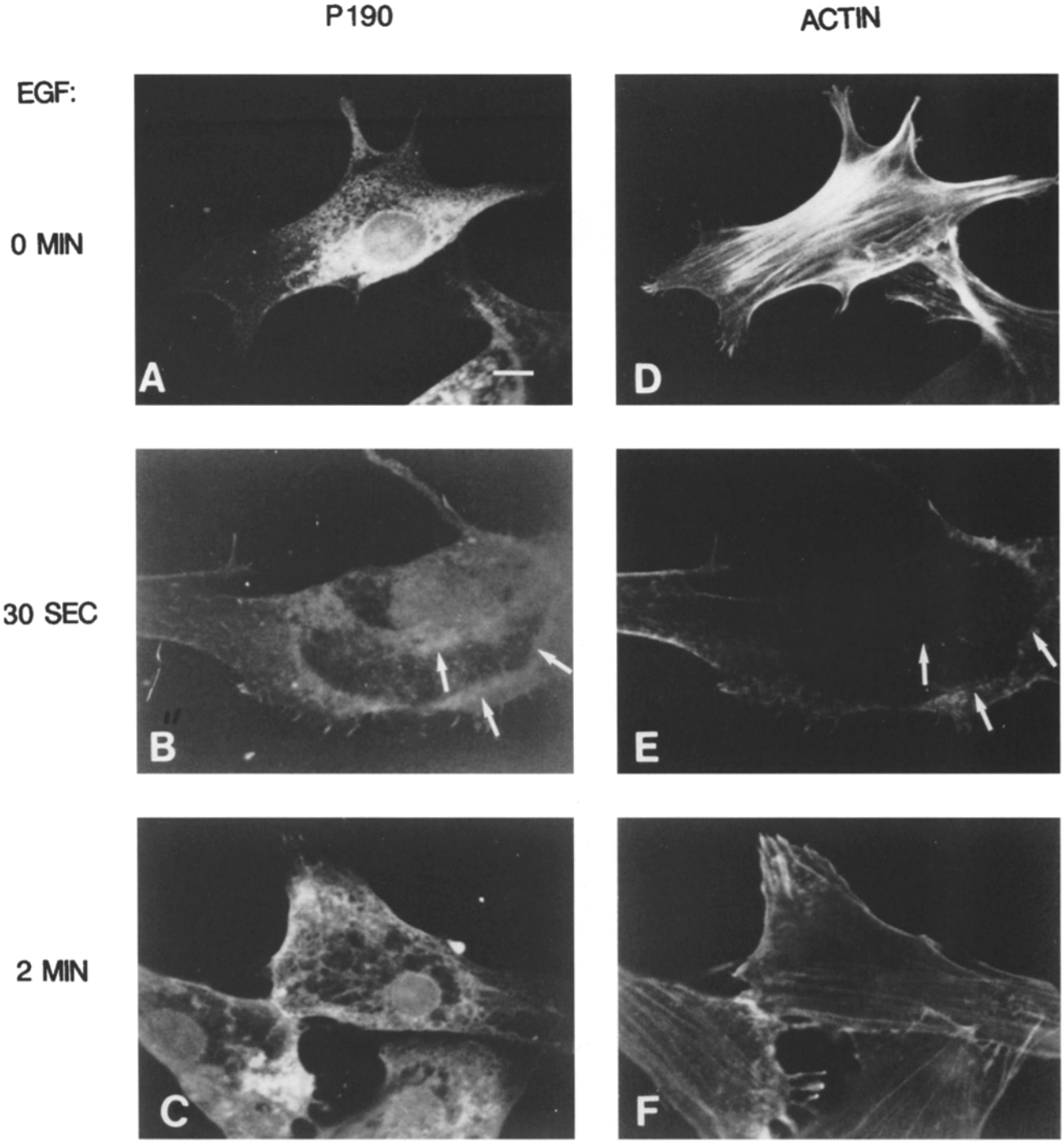

Figure 7. Partial codistribution of p190 and actin after EGF stimulation. K^+ cells were starved of serum, mock-stimulated, or stimulated with 100 ng/ml EGF for 30 s or 2 min, and double-stained for p190 and actin with α-p190 mAb 3D4 and fluorescein-conjugated phalloidin. To detect mAb binding, cells were treated with goat α-mouse IgG and Texas red–conjugated donkey α-goat IgG. Images were recorded by confocal microscopy. p190 and actin colocalized at discrete sites along p190 arcs. The position of these arcs coincided with the boundary between complete vs. partial F-actin dissolution. Bar, 10 μm.

ment of cortical actin, and the formation of membrane ruffles on the cell surface. However, the molecular mechanisms by which growth factors signal these cytoskeletal rearrangements has not been characterized beyond the demonstration of a requirement for the Rho family of small GTP-binding proteins (41, 51, 53). In this paper we provide evidence for a regulatory role for the c-Src tyrosine kinase and an involvement of the GTPase-activating proteins for Rho and Ras, p190RhoGAP, and p120RasGAP, in EGF-induced actin rearrangements. Transient, EGF-dependent redistribution of p190RhoGAP, and p120RasGAP was found to correlate both temporally and spatially with EGF-induced actin reorganization. Superimposed on EGF dependency was a further effect of overexpression of c-Src on actin rearrangement, i.e., both the kinetics and number of cells in a population that underwent actin dissolu-

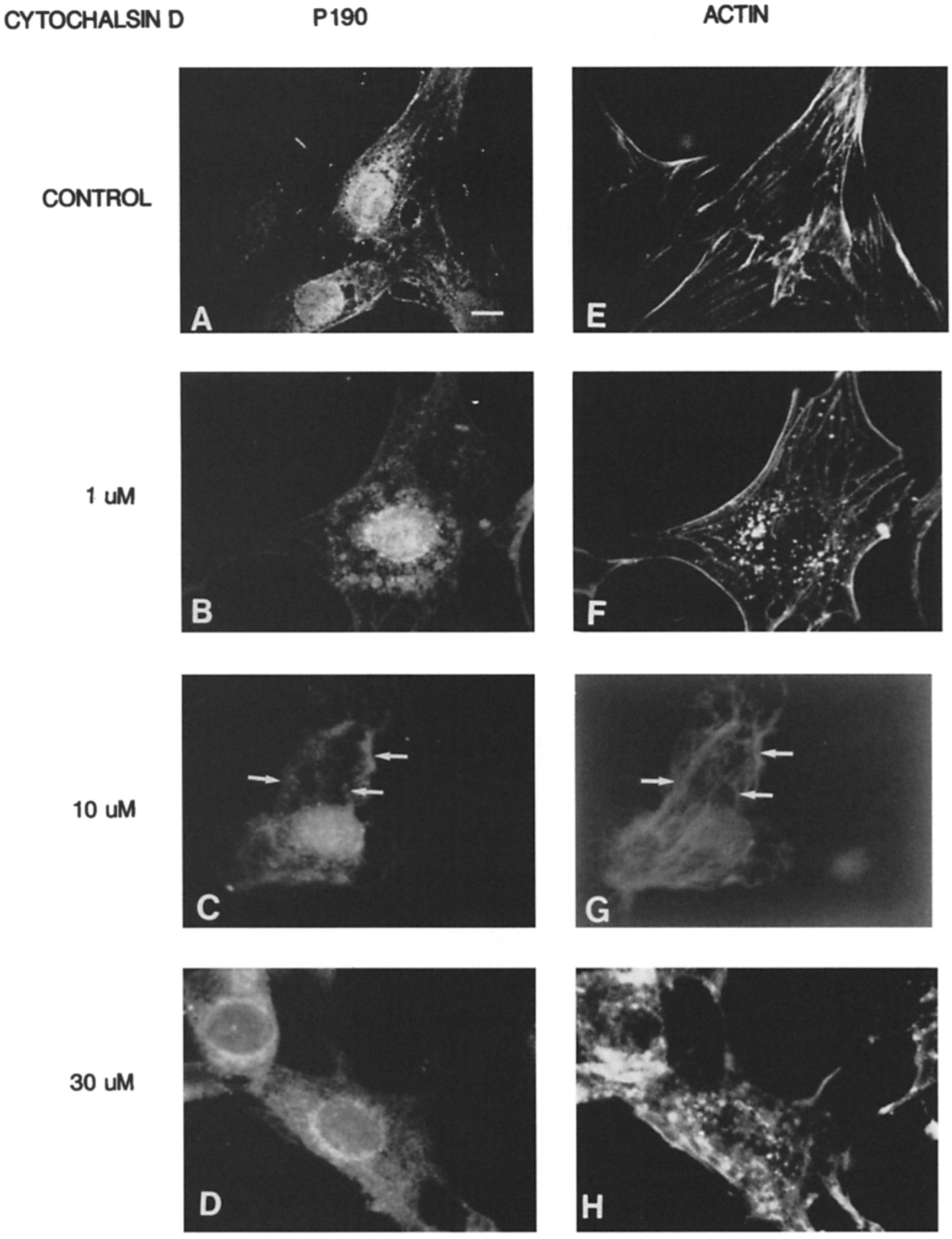

Figure 8. Induction of p190 arc formation by cytochalasin. K^+ cells were starved of serum, incubated with designated concentrations of cytochalasin for 10 min at 37°C, washed, and stained for p190 and actin as in Fig. 7. Images were recorded by confocal microscopy. 10 μM cytochalasin induced the formation of arcs that contained actin (*G*) and p190 (*C*). Bar, 10 μm.

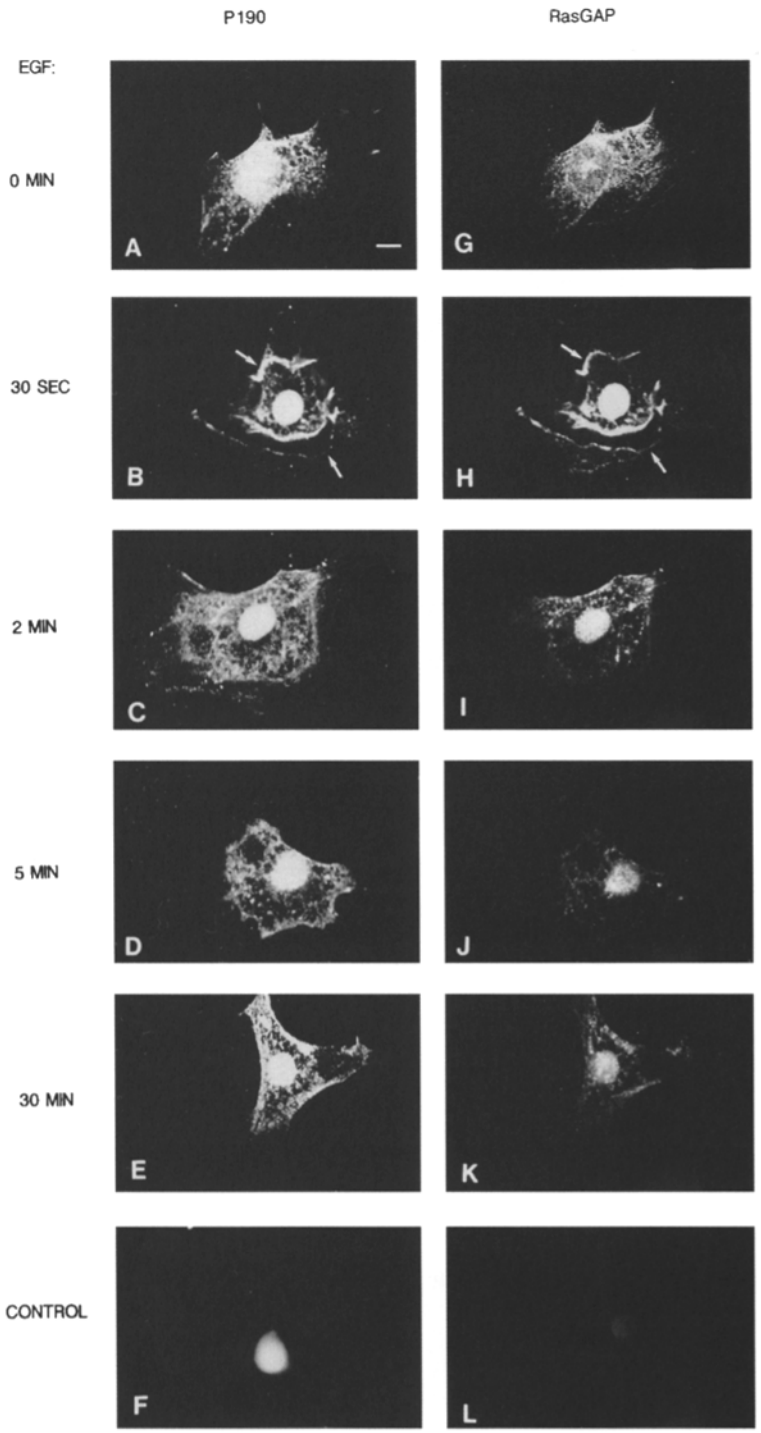

Figure 9. Colocalization of RasGAP with p190 before and after EGF stimulation. K^+ cells were starved of serum and either mock-stimulated or stimulated with 100 ng/ml EGF for the indicated times. Cells in *A–E* and *G–K* were then double-stained for p190 and RasGAP by sequential incubations with affinity-purified rabbit α-p190 plus α-RasGAP mAb 6F2, goat α-rabbit IgG plus rat α-mouse IgG, and Cy3-conjugated donkey α-goat plus Cy5-conjugated donkey α-rat IgG. *F* and *L* lack 1° antibody. Images were recorded by confocal microscopy. Bar, 10 μm.

tion were dependent upon the level and kinase activity of c-Src. Since previous work had identified p190 (and not p120RasGAP) as both a direct substrate of c-Src in 10T1/2 cells (10) and an activator of the intrinsic GTPase activity of Rho family members (54, 59), and Rho as a regulator of actin stress fiber formation, a pathway from c-Src to actin can be deduced (Fig. 10).

In this model (which is only one of a number of possibilities), c-Src functions as a fine tuner of events initiated by EGF, i.e., actin disassembly is regulated by two signals. The primary signal emanates from EGF binding its receptor and the other, an independent signal from c-Src. When the EGF signal is provided, p190RhoGAP and p120RasGAP condense into arclike structures around the nucleus, and actin stress fiber disassembly is initiated. c-Src, through prior phosphorylation of p190, acts to regulate the timing and extent of these events. Since activated, GTP-bound Rho has been shown to induce actin stress fiber formation, it is presumed that actin disassembles when Rho is in the GDP-bound form. The conversion of RhoGTP to RhoGDP, and thus actin from the assembled to the disassembled state is accelerated by members of the RhoGAP family, which includes p190 (32). Elevated tyrosyl phosphorylation of p190 correlates with more rapid and exaggerated

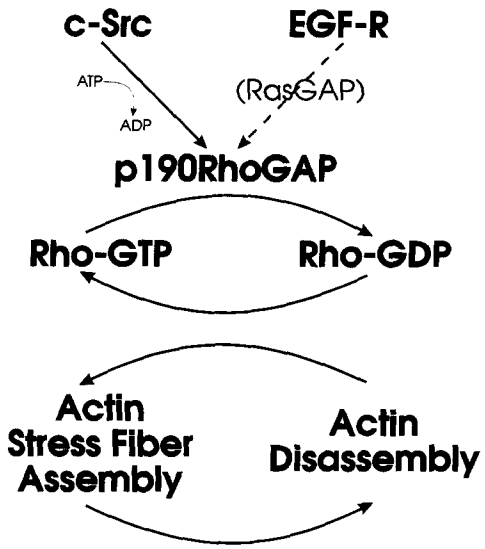

Figure 10. Model for c-Src regulation of actin stress fiber assembly and disassembly. Actin disassembly occurs upon stimulation with EGF. The mechanism of signal transduction from the EGF receptor to actin is unknown, but is depicted as being mediated through RasGAP, since RasGAP and p190RhoGAP are known to form a stable complex. RhoGAP activity is proposed to be regulated by two factors in the 10T1/2 system, by signals from the receptor and by c-Src. Preexisting c-Src–mediated phosphorylation of p190RhoGAP is depicted as promoting hyperactivation of RhoGAP, resulting in a rapid and efficient conversion of RhoGTP to RhoGDP. When less RhoGTP is available, actin is not as actively bundled (or maintained in the filamentous state), resulting in disassembly of the actin cytoskeleton.

disassembly, suggesting that tyrosyl phosphorylation of p190 may increase its RhoGAP activity. This prediction currently is being tested.

In the model depicted in Fig. 10 the initiating signal for actin redistribution is provided by the activated growth factor receptor. However, the nature of this signal is not known. One possibility is a pathway involving p120RasGAP. In this scenario RasGAP could function as an effector of Ras, becoming activated through the receptor-GRB2-SOS-Ras pathway (19). Activated RasGAP might then induce an increase in the RhoGAP activity of p190 (a portion of which is phosphorylated by c-Src and precomplexed with RasGAP [10, 15, 49; and this report]), which in turn could down-modulate RhoGTP, thereby causing a dissolution of actin. Tyrosyl phosphorylation of p190 by c-Src could affect a number of events in this pathway, including the affinity of p190 for RasGAP (8, 40), the affinity of RhoGDI for Rho (22), or the intrinsic GAP activity of p190 for Rho. Tyrosyl phosphorylation may also affect the ability of p190 to form additional complexes containing other cellular proteins. Candidates for such proteins include actin, actin-binding proteins, Rho family members, or upstream transducers of the receptor-initiated signal. In this regard, the GTP-binding domain of p190 could serve as a nucleation site for protein–protein interaction, in a manner similar to p21Ras (14). Indeed, the dynamic and transient nature of the p190 arcs invokes the involvement of a regulatory mechanism such as a GTP/GDP switch (4, 5). The identification of proteins that interact with p190 may provide some clues as to the mechanism of arc formation and its role in actin stress fiber disassembly.

Alternative mechanisms to relay the signal from the receptor to actin include a direct interaction of the receptor with actin (13) and activation of receptor-associated signaling molecules, such as phospholipase Cγ (PLCγ) and phosphatidylinositol-3 kinase (44), that have been implicated in regulating actin-controlling proteins, like gelsolin and profilin, through production of polyphosphoinositides and release of intracellular calcium (20, 45, 48, 63). In addition, Peppelenbosch et al. (47) have demonstrated that EGF-induced actin polymerization/depolymerization can be regulated by leukotrienes and prostaglandins, metabolites of arachidonic acid. These agents have also been shown to regulate the activity of RhoGAPs in vitro (64), suggesting that they could function by interacting with p190 directly. Finally, recent studies using tyrosine kinase inhibitors suggest that focal adhesion kinase (24, 57) may mediate actin remodelling after lysophosphatidic acid stimulation (52). Thus, multiple pathways may transmit signals to the actin cytoskeleton from the receptor, but the existing evidence suggests that p190 is a significant, if not a major, player in this process.

Relationship between Arc Formation, Actin Reorganization, and Mitogenesis

The level of p190 tyrosyl phosphorylation and the kinetics/extent of actin dissolution after EGF treatment correlate with the effects of c-Src on DNA synthesis, i.e., the amount of [^{3}H]thymidine uptake in response to EGF is proportional to the level of active c-Src kinase in the cells. However, in the 10T1/2 system, stress fiber depolymerization and DNA synthesis are not temporally related events. Actin reorganization occurs within seconds to minutes after EGF treatment, while DNA synthesis begins ~14–18 h after stimulation. Yet both events are influenced by c-Src (33, 70; this study). These considerations raise questions about the relationship between these events and the mechanism of c-Src action on each. Is the mechanism of c-Src action distinct for each event, or does potentiating one event affect the other? How can cytoskeletal rearrangements that occur seconds to minutes after growth factor stimulation have an effect on later events like DNA synthesis? What is the physiological significance of the arcs?

Considering the possibility that growth factor–induced actin remodeling may not be directly related to events that culminate in DNA synthesis and proliferation, c-Src would most likely phosphorylate or interact with a distinct set of proteins for each process. For example, p190 would be a likely c-Src target for its effect on cytoskeletal changes, and the EGF receptor (through GRB-2/SHC-SOS- and Ras) a target for DNA replication (34). Alternatively, p190 arc formation, actin rearrangements, and DNA synthesis may be causally linked, and a number of roles for arc formation can be envisioned. First, translocation of p190 into concentric arcs may be necessary for its ability to associate with Rho and activate its GTPase activity. Second, the concentric arc configuration may serve to colocalize other signaling molecules for appropriate interactions. For example, p190 might serve as a transporter for bringing cytosolic serine/threonine kinases (which can be activated by

a number of receptor-mediated pathways) into juxtaposition with perinuclear components such as transcription factors. Examples of such kinases are the MAP kinase family, protein kinase C, cAMP-dependent protein kinase, casein kinase II, rel, and calcium/calmodulin–dependent kinases, all of which in a variety of paradigms have been demonstrated to phosphorylate transcription factors and in most cases to modulate their activities (1, 2, 21, 27, 29, 36, 46). The time frame in which arc formation occurs is compatible with immediate early signaling. Finally, actin rearrangement and arc formation could allow interaction of signal transduction components with cyclins, an event that might serve as an early checkpoint in the cell cycle. Thus, arc formation could signify the assembly and interaction of many intracellular components involved in the early events of mitogenesis.

The point in the cell cycle wherein this process occurs might be critical to subsequent progression through the cell cycle. The check point under consideration appears to occur at the G0/G1 boundary or in early G1. Just as G0/G1 transitions are productive in a fraction of cells, so are a fraction of cells undergoing actin rearrangements. Since overexpression of c-Src appears to widen the window for passage through this putative check point, a greater number of cells in a population may successfully transit this phase of the cycle and progress through G1 and S. The opposite would be true of cells overexpressing kinase defective c-Src, where arc formation is delayed and actin dissolution is less striking. Furthermore, fewer cells in a population exhibit well-formed arcs. This scenario is also consistent with our previous studies which demonstrate that overexpression of c-Src results in enhanced DNA synthesis in response to EGF (33). More cells in a population respond to EGF, when compared with control cells. Conversely, expression of kinase defective c-Src greatly reduces the number of cells progressing to S phase (70). Thus, the kinetics and extent of arc formation correlate well with the ability of a cell to progress through the cell cycle. These considerations prompt the speculation that at least one mechanism by which c-Src could influence growth is as a competence factor, which prepares the cell to receive growth factor signals in a productive manner.

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