JCB Report

Active Rho is localized to podosomes induced by oncogenic Src and is required for their assembly and function

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Transformation of fibroblasts by oncogenic Src causes disruption of actin stress fibers and formation of invasive adhesions called podosomes. Because the small GTPase Rho stimulates stress fiber formation, Rho inactivation by Src has been thought to be necessary for stress fiber disruption. However, we show here that Rho[GTP] levels do not decrease after transformation by activated Src. Inactivation of Rho in Src-transformed fibroblasts by dominant negative RhoA or the Rho-specific inhibitor C3 exoenzyme disrupted podosome structure as judged by localization of podosome components F-actin, cortactin, and Fish. Inhibition of Rho strongly inhibited Src-induced proteolytic degradation of the extracellular matrix. Furthermore, development of an in situ Rho[GTP] affinity assay allowed us to detect endogenous Rho[GTP] at podosomes, where it colocalized with F-actin, cortactin, and Fish. Therefore, Rho is not globally inactivated in Src-transformed fibroblasts, but is necessary for the assembly and function of structures implicated in tumor cell invasion.

Introduction

Regulation of actin dynamics, cell-substrate adhesion, and ECM remodeling is key to the invasive capacity of oncogenically transformed cells. Transformation of fibroblasts in culture by activated mutants of Src, either v-Src or c-Src (Y527F), provides a well-characterized model system for study of regulatory mechanisms involved in tumorigenesis. Oncogenic Src regulates actin bundling and capping proteins and focal adhesion proteins, affects integrin function, and promotes expression and secretion of matrix metalloproteinases (Frame, 2004). Active Src also promotes the formation of podosomes (Tarone et al., 1985), specialized structures at the cell-substrate interface similar to the invasive structures found in macrophages and osteoclasts (for review see Linder and Aepfelbacher, 2003). Podosomes contain a complex array of actin and adhesion regulatory proteins including F-actin, Src, FAK, PI(3)-kinase, integrins, cortactin, vinculin, N-WASP, p190RhoGAP, and Fish (Abram et al., 2003; Linder and Aepfelbacher, 2003). Membrane-bound and secreted matrix metalloproteinases are also localized to podosomes, resulting in concentration of extracellular proteolytic activity at these sites. Individual podosomes often accumulate in ring-like structures termed rosettes (Chen, 1989).

The Rho family GTPases RhoA and Cdc42, well-known for controlling actin dynamics (Hall, 1998), have been implicated in podosome formation in osteoclasts and endothelial cells, respectively (Chellaiah et al., 2000b; Moreau et al., 2003). Rho activity is regulated upon adhesion to ECM proteins (Ren et al., 1999), a process under the control of Src family kinases (Klinghoffer et al., 1999). In addition, Rho GTPases are regulated during polarized movement of fibroblasts controlled by Src (Timpson et al., 2001). The original observation that oncogenic Src causes actin stress fiber loss has lead to the widely held hypothesis that Rho or Rho-dependent signaling is inhibited in transformed cells (Frame et al., 2002). This hypothesis has been supported by the observations that overexpression of constitutively active mutants of Rho can cause restoration of actin stress fibers in Src-transformed fibroblasts (Fincham et al., 1996; Mayer et al., 1999). However, here we show that endogenous Rho is not inactivated in Src-transformed cells. Instead, active Rho is necessary for Src-induced podosome assembly and function.

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The online version of this article contains supplemental material.

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Key words: oncogene protein pp60(v-src); neoplastic cell transformation; rho GTP-binding proteins; cell membrane protrusions; signal transduction

Abbreviations used in this paper: RBD, Rho binding domain; ts-v-Src, temperature-sensitive v-Src.

The Journal of Cell Biology, Volume 166, Number 3, August 2, 2004 317–323
http://www.jcb.org/cgi/doi/10.1083/jcb.200312168

The Journal of Cell Biology

Inhibition of Rho by either expression of dominant negative RhoA or treatment with C3 exoenzyme perturbs both podosome structure and function. Rho inhibition leads to disruption of F-actin, and disruption of cortactin and Fish localization at podosomes, accompanied by a dramatic decrease in protease secretion as measured by in situ zymography. We developed a high resolution in situ Rho[GTP] affinity assay, which revealed that Rho[GTP] is enriched in podosomes, where it colocalizes with F-actin, cortactin and Fish. These findings suggest dynamic regulation of Rho in Src-transformed fibroblasts.

Results and discussion

To directly test the hypothesis that Rho is inactivated in fibroblasts transformed by oncogenic Src, we used a Rho affinity precipitation assay, which takes advantage of the selective interaction of the Rho binding domain (RBD) of the effector rhotekin with active Rho[GTP] (Ren et al., 1999). As expected, GST-RBD selectively precipitated transfected constitutively active Rho, but not dominant negative Rho, as well as endogenous Rho[GTP] from Swiss3T3 cells stimulated with serum (unpublished data). The rhotekin RBD binds both RhoA and the highly related family member RhoC (Reid et al., 1996). With commercial antibodies, we have not observed a difference between the family members in biochemical assays, so we refer to both activities collectively as Rho.

We detected no decrease in Rho[GTP] levels in v-Src-transformed NIH3T3 cells compared with nontransformed control cells growing in the presence of serum under either sub-confluent or confluent conditions (Fig. 1 A), in agreement with a previous report on Src-transformed rat cells (Pawlak and Helfman, 2002). Similarly, there was no difference in endogenous Rho[GTP] levels in NIH3T3 cells transformed with oncogenic c-Src(Y527F) relative to control cells (Fig. 1 B). Activation of temperature-sensitive v-Src (ts-v-Src) did not cause a decrease in Rho[GTP] (Fig. 1 C, lanes 1–7), though Src kinase activity was induced within 1 h (unpublished data). Instead, 6 h after activation of ts-v-Src we observed a modest but reproducible activation of Rho (Fig. 1 C, lanes 8–9).

Our observation that endogenous Rho[GTP] levels are not reduced in Src-transformed cells prompted us to examine whether Rho function might be required for some aspect of oncogenic transformation. To test this hypothesis, we transiently transfected dominant negative RhoA(S19N) into NIH3T3 cells expressing c-Src(Y527F). Staining for F-actin revealed that dominant negative Rho had striking effects on the actin cytoskeleton (Fig. 2, C and G). Transfected cells generally became flattened and more spread, and the cell shape changes were accompanied by an overall loss of actin polymerization. The effect on the actin cytoskeleton was most pronounced at the podosome rosettes, which appeared to be totally or partially disorganized. Expression of dominant negative RhoA(S19N) had a similar effect on polymerized actin in several different clones of NIH3T3 fibroblasts expressing v-Src, although in those cells podosomes appear as small dot-like structures instead of rosettes (unpublished data). Transient overexpression of RhoA(Q63L) either caused

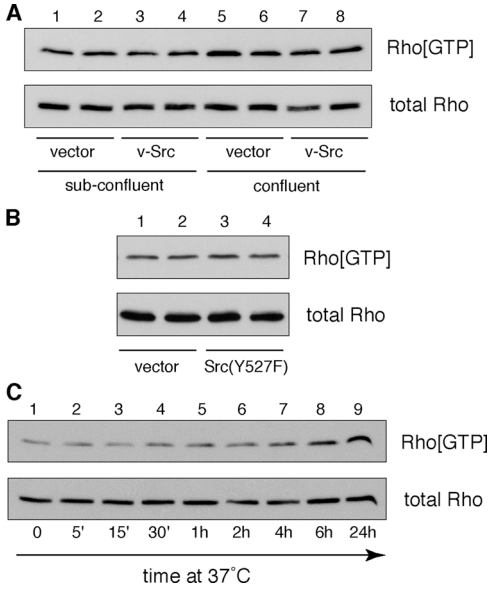

Figure 1. **Endogenous Rho is active in cells expressing oncogenic Src.** (A–C) Rho affinity pull-down assays were used to determine the level of active Rho. In each panel, an anti-RhoA/B/C antibody was used to visualize Rho[GTP] in pellet fractions (top blot) or total Rho in 5% of the cell lysate (bottom blot). (A) Rho activity in duplicate samples of control (vector, lanes 1, 2, 5, and 6) and v-Src–transformed (lanes 3, 4, 7, and 8) NIH3T3 fibroblasts growing in 10% FCS under subconfluent (lanes 1–4) or confluent conditions (lanes 5–8). (B) Rho[GTP] levels in duplicate lysates from NIH3T3 fibroblasts expressing empty vector (lanes 1–2) or c-Src(Y527F) (lanes 3–4). (C) Rho pull-down assays of lysates from subconfluent cultures of NIH3T3 cells expressing ts-v-Src(*tsLA90*). Cells were serum-starved for 20 h at 39.5°C (lanes 1–8) or at 37°C (lane 9) and then shifted to 37°C for the indicated times.

stress fiber formation in some cells, as reported previously (Fincham et al., 1996; Mayer et al., 1999), or in the majority of cells induced extreme cell rounding accompanied by a cortical ring of actin (unpublished data).

To exclude the possibility that overexpression of RhoA (S19N) exerts nonspecific effects on other Rho-related GTPases, we used recombinant Tat-C3 exoenzyme (Sahai and Marshall, 2003) to inhibit Rho. Tat-C3 enters cells via receptor-mediated endocytosis and specifically ADP-ribosylates and inhibits Rho (Sekine et al., 1989). Endogenous Rho was efficiently modified by the inhibitor, because all of the detectable Rho underwent a mobility shift in polyacrylamide gels (unpublished data). In most of the Src-transformed cells, Tat-C3 exerted similar effects to those exerted by dominant negative Rho. The cells flattened dramatically and had irregular plasma membrane extensions (Fig. 2, compare A with B and compare E with F). In addition, actin polymerization was disrupted at rosettes in c-Src(Y527F) cells (Fig. 2, B and F) and at individual podosomes in v-Src cells (unpublished data). Inhibition of Rho in Src-transformed cells did not cause apoptosis, as judged by DAPI staining (Fig. 2, I–K).

Because RhoA has been implicated in podosome formation in osteoclasts (Chellaiah et al., 2000b) and dendritic cells (Burns et al., 2001), we further investigated the effect of Rho inhibition on podosome integrity by immunostaining

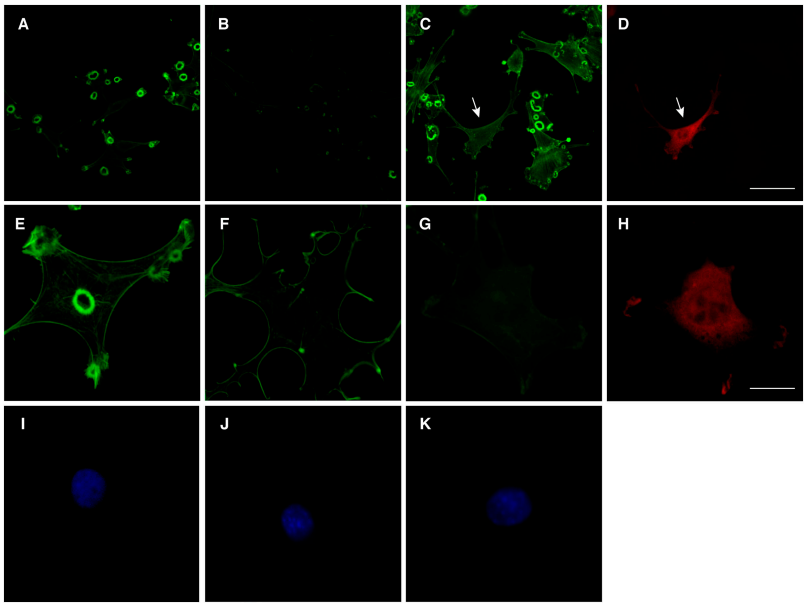

Figure 2. **Inhibition of Rho disrupts F-actin in Src-transformed fibroblasts.** Src(Y527F) fibroblasts exponentially growing on glass were left untreated (A, E, and I) or treated with 0.5 μM Tat-C3 for 18 h (B, F, and J) and processed for immunofluorescence. Another set of Src(Y527F) fibroblasts were transfected with myc-RhoA(S19N) for 24 h, and plated on glass for an additional 12 h (C, D, G, H, and K) before processing for immunofluorescence. (A–C) F-actin staining at low magnification. (D) myc costaining corresponding to field in C. Arrows in C and D indicate transfected cell. (E–H) High magnification images of representative cells from the same experiments. (E–G) F-actin; (H) myc. G and H correspond to the same cell. (I–K) DAPI staining of corresponding nuclei in E–G. Bars: (D) 30 μm; (H) 9.5 μm.

for two different podosome components: the Src substrates cortactin (Okamura and Resh, 1995) and Fish (Abram et al., 2003). Consistent with the loss of polymerized actin, we did not detect cortactin or Fish at podosome rosettes after treatment of Src(Y527F) cells with Tat-C3 (Fig. 3, compare A with C and compare B with D). Similarly, expression of dominant negative Rho disrupted cortactin localization to rosettes in Src(Y527F) cells (Fig. 3 E).

Matrix proteases are localized to podosomes and rosettes (Linder and Aepfelbacher, 2003). MMP-2 secretion in fibroblasts transformed by v-Src is dependent on a v-Src-FAK-JNK pathway (Hauck et al., 2002). Interestingly, RhoA- and JNK-dependent pathways reportedly interact to influence MMP-9 secretion in keratinocytes (Turchi et al., 2003). To investigate the functional significance of podosome loss after Rho inhibition, we asked whether Rho function is necessary for Src-induced ECM degradation. Src-transformed fibroblasts were cultured on cross-linked gelatin conjugated to a fluorescent dye, and protease secretion was indicated by formation of small nonfluorescent patches beneath the cells (Fig. 4, A and D). As expected, these patches very often colocalized with F-actin (Fig. 4, G–I). Treatment of Src(Y527F)-expressing cells with Tat-C3 almost completely abolished protease secretion compared with untreated cells (Fig. 4, A, B, and J). In contrast, pharmacological inhibition of the Rho effector Rho-kinase with Y-27632 did not significantly affect gelatin degradation (Fig. 4, C and J), suggesting that the effect of Rho on podosomes is not mediated by the Rho-kinase pathway. Interestingly, Sahai and Marshall (2003) have recently demonstrated that a "blebbing" mode of tumor cell invasion requires the Rho–ROCK pathway but not pericellular proteolysis, suggesting Rho can regulate cell invasion by different mechanisms in different cellular contexts. We also found that expression of constitutively active RhoA(Q63L) inhibited Src-induced gelatin degradation (Fig. S1, G–J, available at http://www.jcb.org/cgi/content/full/jcb.200312168/DC1), but to a lesser extent than dominant negative RhoA(S19N) (Fig. S1, A, D, and J). It is possible that the effects of RhoA(Q63L)

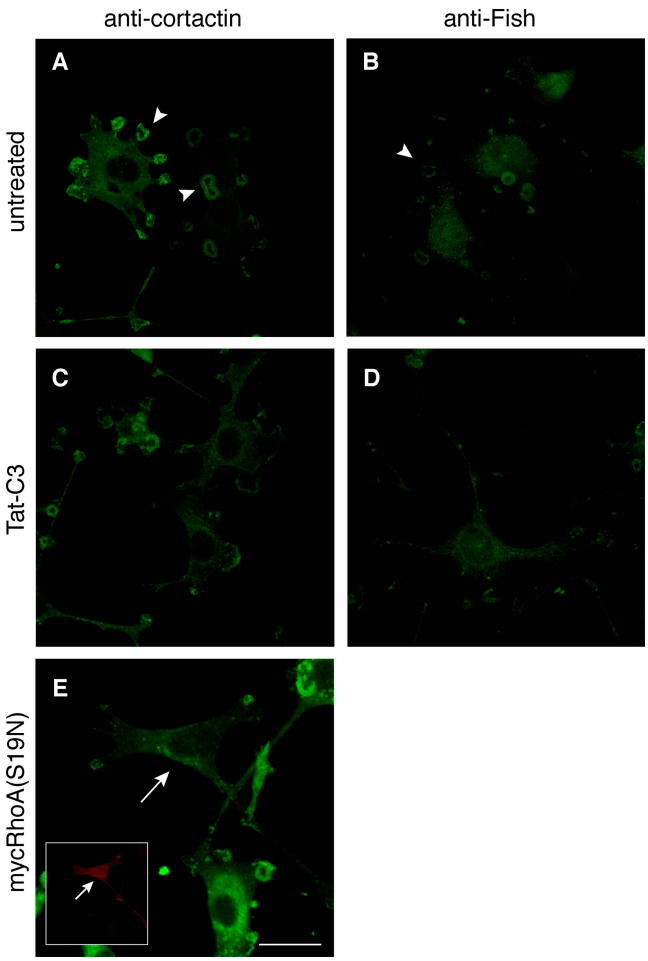

Figure 3. **Inhibition of Rho in Src-transformed fibroblasts causes podosome disassembly.** Mouse fibroblasts expressing Src(Y527F) exponentially growing on glass were left untreated (A and B) or treated with 0.5 μM Tat-C3 for 18 h (C and D). Another set of Src(Y527F) fibroblasts were transfected with myc-RhoA(S19N) for 24 h, and plated on glass for an additional 12 h (E). Samples were processed for immunostaining for cortactin (A, C, and E) or Fish (B and D). Arrowheads in A and B indicate podosome rosettes. Inset in E shows myc costaining of the cell in E (arrows). Bar, 17.5 μm.

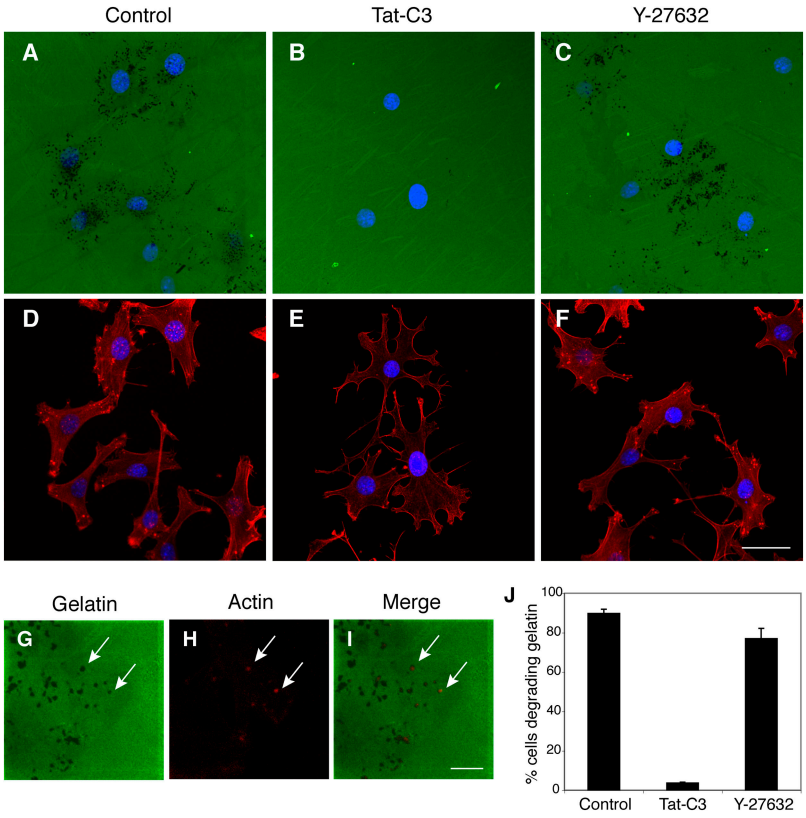

Figure 4. **Inhibition of Rho but not ROCK impairs Src-induced protease secretion.** Src(Y527F) cells exponentially growing on plastic in complete medium were treated with vehicle (A and D), 0.5 μM Tat-C3 (B and E), or 10 μM Y-27632 (C and F) for 20 h. Cells were then trypsinized and plated on glass coverslips coated with cross-linked Oregon green®–labeled gelatin in medium containing the indicated inhibitor. After 3 h, cells were fixed and stained for F-actin and DAPI. (A–C) Superimposed images of gelatin (green) and DAPI (blue). (D–F) Superimposed images of the same fields in A–C corresponding to F-actin (red) and DAPI (blue). (G–I) High magnification images of untreated control samples in the same experiment. Degradation patches in Oregon green®–labeled gelatin (G, arrows) and F-actin condensation at podosomes (H, arrows) colocalize when red and green channels are merged (I, arrows). (J) Gelatin degradation was quantified and is represented as the percent of cells able to form degradation patches (see Materials and methods). Results are the mean ± SD of three different experiments. Bars: (F) 32 μm; (I) 6 μm.

may be due, at least in part, to the rounding that occurs in the majority of the transfected cells and the consequent decrease in cell-substratum contact area. However, these findings suggest that cycling of Rho between GTP-bound and GDP-bound states may be important for matrix degradation induced by oncogenic Src.

Localization of the Src substrate and Rho regulator p190RhoGAP to invadopodia in metastatic melanoma cells (Nakahara et al., 1998) and to podosomes in Src-transformed cells (Gavazzi et al., 1989) raises the possibility of spatially restricted regulation of Rho at those sites. Therefore, we developed an in situ Rho[GTP] affinity assay to determine the subcellular distribution of endogenous active Rho. Fixed cells were incubated with purified soluble GST-RBD, and specific binding was detected by anti-GST staining. While this manuscript was in preparation, a similar assay was described (Dubreuil et al., 2003). As in the affinity precipitation assay, constitutively active RhoA(Q63L) was strongly recognized by soluble GST-RBD (Fig. S2, B and E, available at http://www.jcb.org/cgi/content/full/jcb.200312168/DC1). Soluble GST-RBD-AAA (see Online supplemental material), which contains point mutations that diminish binding to Rho[GTP] (Fig. S2 A and Fig. S3 A, available at http://www.jcb.org/cgi/content/full/jcb.200312168/DC1), recognized overexpressed RhoA(Q63L) to a much lesser extent, and GST alone yielded an almost undetectable signal (Fig. S2, compare B–D), confirming the specificity of the assay.

We were also able to use soluble GST-RBD to detect endogenous Rho[GTP] in Src-transformed fibroblasts (Fig. S3, B and E). As with transfected mutants of Rho, endogenous Rho[GTP] was specifically detected by wild-type GST-RBD. In matched controls, the mutant GST-RBD-AAA showed reduced binding, and GST alone yielded negligible staining (Fig. S3, B–D). Rho[GTP] appeared at various ventral adhesions and membrane ruffles in nontransformed fibroblasts (unpublished data). Strikingly, with this assay we observed an enrichment of Rho[GTP] at podosome rosettes in cells expressing Src(Y527F) (Fig. 5, A, D, and G), where it colocalized with cortactin (Fig. 5, B and C), Fish (Fig. 5, E and F), and actin (Fig. 5, H and I). High magnification images show that Rho[GTP] is concentrated at individual podosomes in a pattern similar to that of F-actin (Fig. S3, compare E with F). As a further confirmation of the specificity of the in situ affinity assay for Rho[GTP], no localized Rho[GTP] was detectable after Tat-C3 treatment, even at sites where residual F-actin was still detectable at partially disrupted podosome rosettes (Fig. 5, J–L). These data demonstrate for the first time that endogenous active Rho is concentrated at sites of actin remodeling and ECM invasion in oncogenically transformed cells.

Podosomes are dynamic structures that are continually assembled and rearranged on time scales of the order of minutes. Our finding that Rho[GTP] is concentrated in podosomes (Fig. 5) raises the possibility that Rho activation occurs at these sites. FRET-based analysis of Rac activation has revealed that membrane recruitment of Rac1 and release of RhoGDI are restricted spatially to sites of integrin engagement (Del Pozo et al., 2002). It is conceivable that Rho is activated in an analogous manner because podosomes contain numerous proteins, including FAK, that could activate or recruit Rho-specific guanine nucleotide exchange factors (Hauck et al., 2002; Zhai et al., 2003). In addition, Src

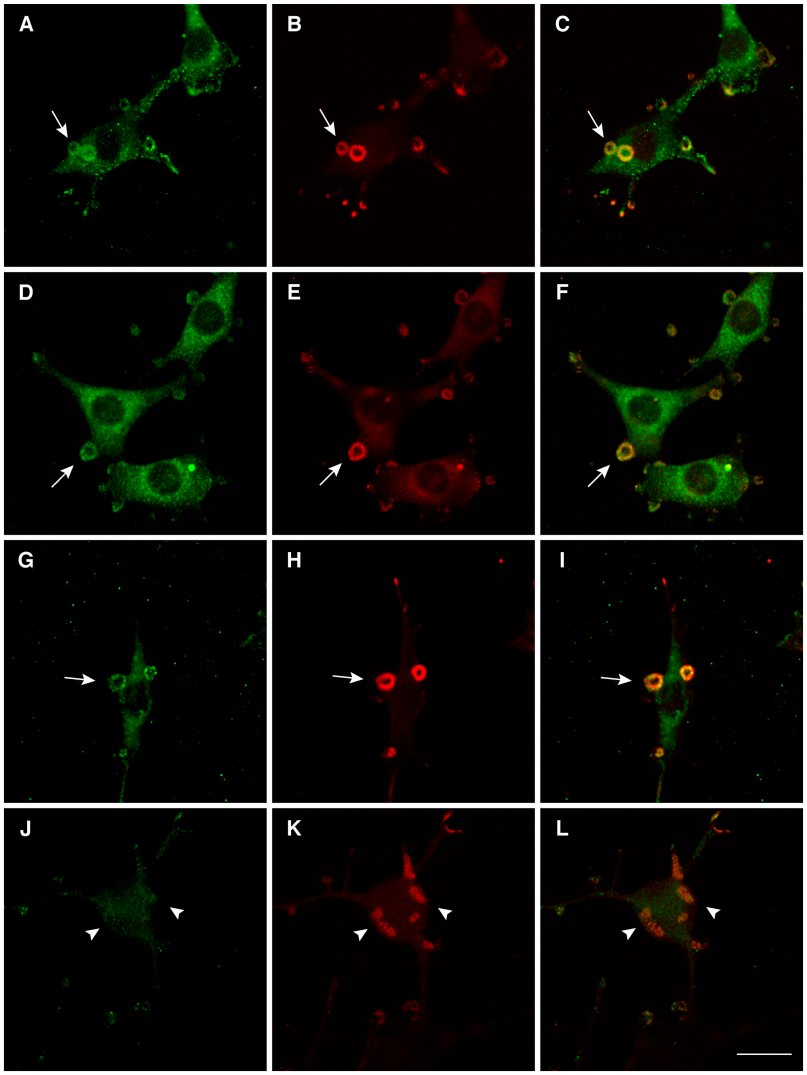

Figure 5. **Active Rho[GTP] at podosome rosettes colocalizes with podosome components.** Src(Y527F) cells exponentially growing on glass were left untreated (A–I) or were treated with 0.5 μM Tat-C3 for 18 h (J–L). After fixation, cells were incubated with 50 μg/ml of soluble GST-RBD. Rho[GTP]-bound RBD was detected by GST immunostaining (A, D, G, and J, green). The same samples were costained for the podosome components cortactin (B), Fish (E), or F-actin (H, control; K, Tat-C3), red. Merged green (Rho[GTP]) and red (podosome component) channels are shown in C, F, I, and L (yellow). Arrows in A–I indicate rosettes; arrowheads in J–L indicate disrupted rosettes. Bar, 14 μm.

could activate an exchange factor directly by tyrosine phosphorylation (Chikumi et al., 2002). Based on our in situ Rho[GTP] assay in fixed cells, we cannot determine whether Rho is activated at podosomes or activated elsewhere and subsequently recruited. p190RhoGAP phosphorylation by v-Src strongly correlates with the onset of morphological transformation (Fincham et al., 1999). Given the reported localization of p190RhoGAP to invasive structures and our observation that both constitutively active and dominant negative RhoA inhibit podosome function (Fig. S1), it is tempting to speculate that active Src stimulates both GTP hydrolysis and GDP-GTP exchange on Rho at podosomes.

Rho[GTP] might stimulate podosome formation through phosphatidylinositol-4-phosphate 5-kinase-dependent production of PIP_2 (Chong et al., 1994), which activates the actin capping and severing protein gelsolin, an obligate podosome component in osteoclasts (Chellaiah et al., 2000a). Additionally, through its effector mDia1, Rho[GTP] coordinates microtubules, which are important for podosome regulation in macrophages (Linder et al., 2000). Alternatively, it is possible that Rho-dependent actin polymerization is required to maintain localization of activated Src to podosomes, as is the case for trafficking of ts-v-Src to focal adhesions before their degradation (Fincham et al., 1996). Thus, Rho function may be necessary for localization of Src to podosomes, regulation of actin dynamics within podosomes, or both.

We have shown that Rho is not inactivated by oncogenic Src, that Rho function is specifically required for podosome integrity and function, and that endogenous Rho[GTP] is localized to podosome rosettes in Src-transformed cells. Src kinase activity is elevated in many types of human cancers (Frame, 2004), and elevated expression of RhoA and RhoC has been correlated with increased metastatic potential in numerous human tumor cells (Sahai and Marshall, 2002). It will be interesting to determine whether the invasive capacity of tumor cells with elevated Src activity results from Rho-dependent protease secretion.

Materials and methods

In situ zymography

Glass coverslips were prepared essentially as described previously (Mueller et al., 1992). In brief, they were coated with Oregon green® 488-conjugated gelatin (0.2 mg/ml in 2% sucrose buffer), cross-linked 15 min in 0.5% glutaraldehyde in PBS, and incubated for 3 min with 5 mg/ ml $NaBH_4$ in PBS. After quenching with DME at 37°C, cells were plated on

coated coverslips in DME containing 10% FCS with or without inhibitors and incubated at 37°C for 3 or 4 h before processing for immunostaining. Results were quantified by counting cells degrading matrix, as defined by ability to form at least one degradation patch regardless of its size, and represented as a percentage of the total (50 cells per treatment in at least two independent experiments).

In situ Rho[GTP] affinity assay

In situ detection of active Rho was conceptually based on a similar assay developed for detecting Ras[GTP] (Sherman et al., 2000), but adapted for Rho[GTP] and optimized to allow subcellular resolution. Cells growing on glass coverslips were briefly washed in PBS and immediately fixed with freshly prepared 4% PFA in PBS for 10 min RT. After a 30-min incubation in PBS containing 3% BSA, 0.1 M glycine, and 0.05% Triton X-100 followed by 15 min in the same buffer containing 15% goat serum, samples were washed three times in ice-cold wash buffer (0.5% BSA, 0.1 M glycine in PBS) and incubated with the indicated concentrations of soluble GST-RBD, GST-RBD-AAA, or GST for 2 h at 4°C. Samples were washed three times and incubated in wash buffer containing 15% goat serum for 30 min followed by incubation with either monoclonal (1:800) or polyclonal anti-GST (1:500) in wash buffer containing 5% goat serum for 1 h at RT. For costaining, samples were incubated with both primary antibodies overnight at 4°C. Signal was detected by incubation with biotin anti–mouse or anti–rabbit IgG (1:200) for 45 min RT followed by a 30-min incubation with streptavidin Alexa Fluor® 488 with or without rhodamine-phalloidin to detect F-actin (1:100). Samples were mounted in SlowFade® antifade reagent containing DAPI. (See Online supplemental material for further details.) Images were obtained with a Zeiss510 LSM.

Online supplemental material

The Materials and methods section contains additional details including the origin and production of stable cell lines, sources of antibodies and reagents, generation of DNA constructs, methods of biochemical analyses (GTPase pull-down assays, purification of recombinant proteins), and conditions used for immunocytochemistry and imaging.

Fig. S1 shows that dominant negative Rho, and to a lesser extent constitutively active Rho, both inhibit gelatin degradation by Src-transformed fibroblasts. Fig. S2 shows that recombinant GST-RBD specifically recognizes constitutively active Rho(Q63L) in vitro and in situ, whereas mutant GST-RBD-AAA has reduced affinity for constitutively active Rho in vitro and in situ. Fig. S3 further demonstrates the specificity of GST-RBD for endogenous active Rho in vitro and in situ. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200312168/DC1.

The authors are grateful to Chris Marshall (Institute of Cancer Research, London, UK), Martin Schwartz (University of Virginia, Charlottesville, VA), Sara Courtneidge (Van Andel Research Institute, Grand Rapids, MI), Joan Brugge (Harvard Medical School, Boston, MA), and Rong-Guo Qiu (University of California, Berkeley) for gifts of reagents; David Helfman for communication of unpublished results; and Yoav Henis, Peter Lewis, Consuelo Barroso, and David Drubin for advice. We thank Steve Taylor, Enrique J. de la Rosa, and Nicholas Justice for discussions and critical comments on the manuscript; and Denise Schichnes in the U.C. Berkeley College of Natural Resources Biological Imaging Facility for imaging expertise.

R.L. Berdeaux was supported by a predoctoral fellowship from the Howard Hughes Medical Institute and by an National Research Service Award training grant (CA09041). This work was supported by National Institutes of Health grant CA17542 and a Laboratory Directed Research and Development grant from the Lawrence Berkeley National Laboratory to G.S. Martin.

Submitted: 23 December 2003
Accepted: 9 June 2004

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