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Local signaling by the EGF receptor

Stephan J. Kempiak,[1] Shu-Chin Yip,[2] Jonathan M. Backer,[2] and Jeffrey E. Segall[1]

[1]Department of Anatomy and Structural Biology and [2]Department of Molecular Pharmacology, Albert Einstein College of Medicine, New York, NY 10461

Differing spatial scales of signaling cascades are critical for cell orientation during chemotactic responses. We used biotin EGF bound to streptavidin-coupled magnetic beads to locally stimulate cells overexpressing the EGF receptor. We have found that EGF-induced actin polymerization remains localized even under conditions of receptor overexpression. Conversely, EGF-induced ERK activation spreads throughout the cell body after EGF bead stimulation. The localized actin polymerization is independent of PI3-kinase and rho protein activity and requires Arp2/3 complex and cofilin function. Thus, we find differing spatial scales of signaling from the EGF receptor, supporting models of chemotaxis that integrate short- and long-range signaling.

Introduction

Chemotactic responses in amoeboid cells consist of localized extension of actin-rich protrusions such as filopods or lamellipods on the side of the cell closest to the source of chemoattractant, combined with the retraction of the side of the cell farthest away from the source, resulting in translocation up the gradient. Chemotactic responses can be mediated by both G protein–coupled receptors and receptor tyrosine kinases such as the EGF receptor (Parent and Devreotes, 1999; Wells, 2000; Condeelis et al., 2001). Previous studies of intracellular signaling responses to localized application of EGF have identified global signaling responses (Verveer et al., 2000; Sawano et al., 2002), which suggested that overexpression of the EGF receptor delocalizes signaling, and should eliminate chemotaxis. Paradoxically, cells expressing high levels of receptor are still chemotactic (Malliri et al., 1998; Rabinovitz et al., 1999; Bailly et al., 2000). Thus, there must be signals that remain localized even at high receptor density. We report here that actin polymerization induced by the EGF receptor remains localized even in cells expressing high levels of EGF receptor and is dependent upon cofilin and Arp2/3 complex function.

Results and discussion

We initially used metastatic rat mammary adenocarcinoma cells (MTLn3-EGFR) overexpressing the EGF receptor ($>10^5$ EGFR per cell) to identify localized cellular responses. MTLn3-EGFR cells are chemotactic to spatial gradients of EGF, generating actin-rich lamellipods that extend toward higher concentrations of EGF (Bailly et al., 2000). We have used streptavidin-coupled magnetic beads bound to biotin-labeled EGF in order to minimize release of EGF from the bead. A magnet was used to pull the beads rapidly onto the surfaces of the cells. By performing time-lapse analysis, we observed two types of cellular responses to the beads (Fig. 1 A; and Video 1, available at http://www.jcb.org/cgi/content/full/jcb.200303144/DC1). The predominant response was the production of a phase-dense ring or ruffling around the bead (beads marked by arrows in Fig. 1 A). However, >30% of the responses were protrusions with the bead at the tip: in the center cell of Fig. 1 A (bead marked by arrowhead), a protrusion in the form of a helix developed over a ten-min period after contacting the cell (see Video 1).

Fixing and staining cells with rhodamine phalloidin at various times after exposure to EGF-coated beads indicated that polymerized actin was concentrated in both types of responses. Fig. 1 B shows an example of a localized F-actin response. There was an increased level of phalloidin staining at the bead site compared with the rest of the actin cortex in the same plane of focus. Fig. 1 C shows a protrusive response: the bead induced a 15-μm tall protrusion that stains with phalloidin (see Videos 2 and 3 for rotations of Fig. 1, B and C). The peak response to the beads occurred 5 min after application of the beads (Fig. 1 D), with most actin polymerization responses gone and the beads internalized by 30 min

To confirm that EGF receptor activity was responsible for the localized response rather than nonspecific clustering of other membrane proteins, MTLn3:EGFR cells were treated with PD153035, a drug that inhibits EGFR kinase activity,

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Address correspondence to J.E. Segall, Department of Anatomy and Structural Biology, Albert Einstein College of Medicine, 1300 Morris Park Ave., New York, NY 10461. Tel.: (718) 430-4237. Fax: (718) 430-8996. email: segall@aecom.yu.edu

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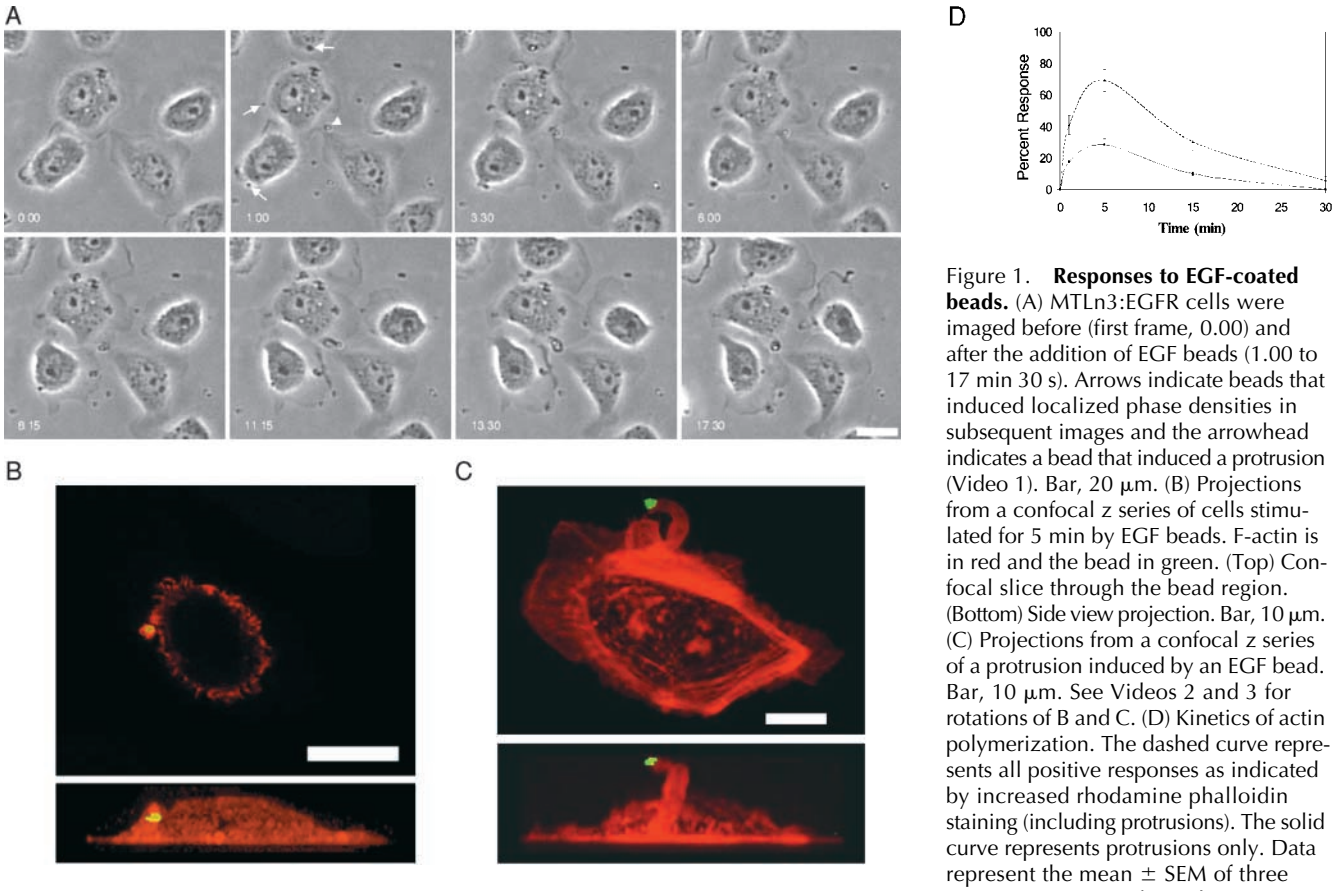

Figure 1. **Responses to EGF-coated beads.** (A) MTLn3:EGFR cells were imaged before (first frame, 0.00) and after the addition of EGF beads (1.00 to 17 min 30 s). Arrows indicate beads that induced localized phase densities in subsequent images and the arrowhead indicates a bead that induced a protrusion (Video 1). Bar, 20 μm. (B) Projections from a confocal z series of cells stimulated for 5 min by EGF beads. F-actin is in red and the bead in green. (Top) Confocal slice through the bead region. (Bottom) Side view projection. Bar, 10 μm. (C) Projections from a confocal z series of a protrusion induced by an EGF bead. Bar, 10 μm. See Videos 2 and 3 for rotations of B and C. (D) Kinetics of actin polymerization. The dashed curve represents all positive responses as indicated by increased rhodamine phalloidin staining (including protrusions). The solid curve represents protrusions only. Data represent the mean ± SEM of three experiments; n = 50 for each experiment. All videos are available at http://www.jcb.org/cgi/content/full/jcb.200303144/DC1.

for 15 min before bead addition. This treatment significantly decreased the number of positive responses (Fig. 2 A), indicating that kinase activity of the EGFR was critical for generating localized responses to beads. Cytochalasin D completely inhibited the EGF bead response, whereas nocodazole treatment had no effect (Fig. 2 A). These data indicate that the increase in filamentous actin around EGF beads is not due to reorganization of preexisting actin but, rather, localized activation of actin polymerization and is independent of microtubule polymerization. The localized response was specific to binding of EGF to the EGFR, because the EGF bead response was completely inhibited when soluble EGF was added at a saturating concentration along with the EGF beads (Fig. 2 B). The soluble ligand stimulated the cell's EGF receptors, as indicated by the rim of filamentous actin at the edge of the cell, but blocked the access of the EGF beads to the receptors.

MTLn3:PLXSN cells expressing 50,000 EGFR/cell (Kaufmann et al., 1994) showed similar responses, but produced half as many protrusions as MTLn3:EGFR cells at 5 min (unpublished data). As a control, nonmetastatic MTC cells, which have very low levels of surface EGFR compared with MTLn3 cells and do not respond to EGF (Segall et al., 1996), did not respond to the EGF beads (unpublished data). We see similar localized actin responses in MTLn3 cells (50,000 EGFR/cell), MTLn3-EGFR cells (~100,000 EGR/cell), MTLn3-EGFR-GFP cells (~250,000 EGFR/cell), MDA-MB-435 EGFR cells (~400,000 EGFR/cell), MCF7 EGFR cells (~1,000,000 EGFR/cell), and A431 cells (~1,700,000 EGFR/cell), indicating that localized signaling to the actin cytoskeleton is independent of EGF receptor density.

Given the published data that EGFR activation can spread throughout the cell (Verveer et al., 2000; Sawano et al., 2002), we wished to identify which signaling pathways remained localized with the filamentous actin response (Fig. 3 A). To determine if the localized response depended on an increased local concentration of EGFR in response to the bead, MTLn3 cells overexpressing an EGFR–GFP fusion protein (Bailly et al., 2000) were used to observe the receptor levels at the bead site. When exposed to EGF beads, GFP fluorescence was not increased at the site of the bead (Fig. 3 A, EGFR:GFP column). Thus, EGF beads could induce a localized response to EGF without increasing the local density of EGFR, although EGF may be released from the bead upon binding to receptor. Both phosphotyrosine staining and phosphoErbB2 were increased near the bead site. When MTLn3 cells are stimulated with EGF, the Arp2/3 complex has been shown to accumulate at the leading edge of a cell (Bailly et al., 2001). N-WASP, an activator of the Arp2/3 complex, and p34, a subunit of the Arp2/3 complex, were both increased near the bead compared with levels at the

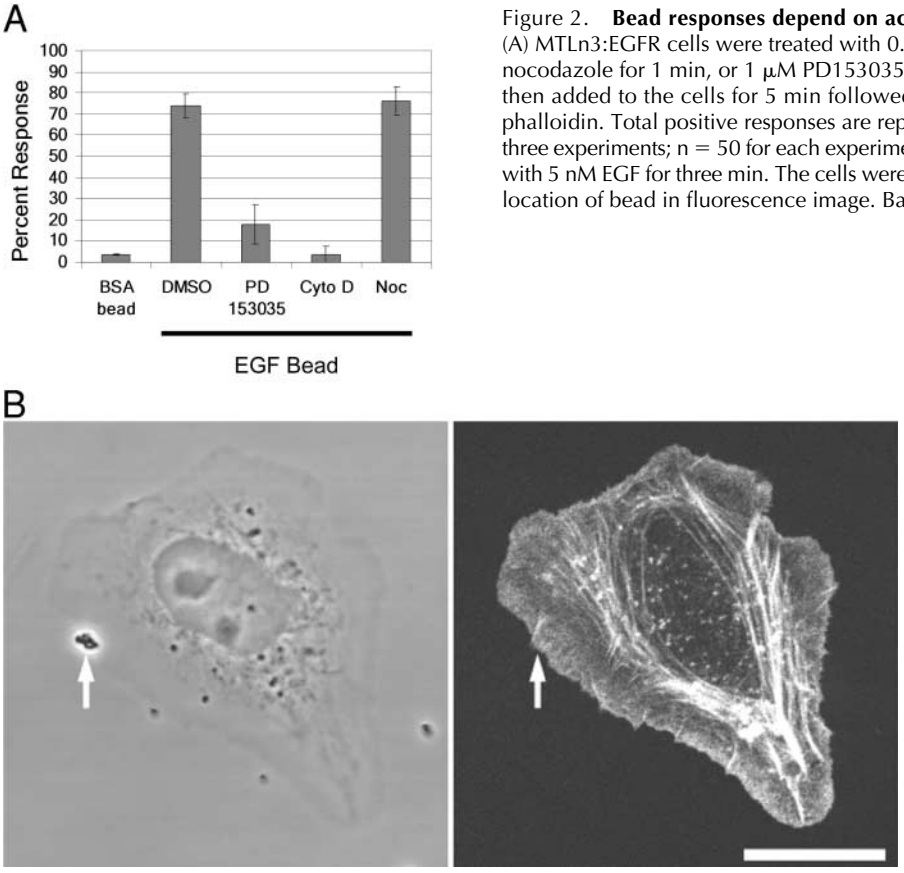

Figure 2. **Bead responses depend on actin polymerization and EGFR kinase.** (A) MTLn3:EGFR cells were treated with 0.1% DMSO, 1 μM cytochalasin D, or 1 μM nocodazole for 1 min, or 1 μM PD153035 for 15 min**.** BSA beads or EGF beads were then added to the cells for 5 min followed by fixation and staining with rhodamine phalloidin. Total positive responses are reported. Data represent the mean ± SEM of three experiments; n = 50 for each experiment. (B) EGF beads were added simultaneously with 5 nM EGF for three min. The cells were stained and fixed for F-actin. Arrow indicates location of bead in fluorescence image. Bar, 20 μm.

edge of the cell. Cofilin, an actin-severing protein, which is near the leading edge of lamellipodia (Chan et al., 2000) also localized to the bead site (Fig. 3 A). These data are consistent with data indicating localized accumulation of shc around EGF beads in A431 cells (Brock and Jovin, 2001). However, FAK and vinculin, proteins associated with focal adhesions (Geiger et al., 2001), did not localize to the source of the bead, arguing against an adhesion or focal contact mechanism for generating the local actin polymerization.

The spatial distance over which signaling pathways were activated was determined by measuring the average radius of increased staining. Phosphotyrosine, phosphoErbB2 and p34 staining all had an average radius of ~1 μm, similar to the size of the beads (Figs. 3, B and C). Polymerized actin and phosphoAkt radii were significantly larger, whereas N-WASP and cofilin staining was more variable in size. PhosphoErk, on the other hand, was increased both at the bead and throughout the cell cytoplasm (Fig. 3 D). Thus, we find a hierarchy of signaling distances, with phosphotyrosine, phosphoErbB2, and p34 being concentrated extremely close to the receptor, polymerized actin and Akt spreading outwards around 0.5 μm farther, and phosphoErk spreading throughout the cell.

Chemotaxing leukocytes and *Dictyostelium* utilize PI3-kinase to polarize toward their respective ligands (Iijima et al., 2002; Weiner et al., 2002). EGF-induced lamellipods are also dependent on PI3-kinase (Hill et al., 2000). It is possible that a positive feedback loop composed of PI3-kinase and rho family proteins could lead to localized amplification of actin polymerization responses. Therefore, MTLn3:EGFR cells were treated with wortmannin, a PI3-kinase inhibitor. There was no decrease in the number of positive responses (determined by phase–contrast or phalloidin staining) with wortmannin treatment (Fig. 4 A, left). As controls, the levels of phosphoAkt induced by either soluble EGF (Fig. 4 B) or EGF beads (Fig. 4 A, right) were measured and found to be decreased in the presence of wortmannin, confirming inhibition of PI3-kinase. Similar results were obtained with MTLn3:PLXSN cells (unpublished data). To determine the contributions of rho family proteins to bead-induced actin polymerization, we treated cells with *C. difficile* toxin B, a potent inhibitor of Rho, Rac, and Cdc42 proteins. Toxin B treatment did not block the EGF bead–induced actin polymerization response (Fig. 4 C), although rac activation by EGF was blocked (Fig. 4 D). Introduction of dominant–negative rac or cdc42 by transfection of cDNA constructs or microinjection of protein also had no effect (unpublished data). Thus, rho family proteins, PI3-kinase, and phosphoAkt are not necessary for generation of a localized actin polymerization response.

To identify the mechanism of localized actin polymerization, we evaluated the roles of cofilin and the Arp2/3 complex. Actin polymerization at the leading edge of MTLn3 cells in response to soluble EGF requires both Arp2/3 complex (Bailly et al., 2001) and cofilin (Chan et al., 2000) activity in vitro as well as in vivo (DesMarais, V., F. Macaluso, J. Condeelis, and M. Bailly, personal communication). We used siRNA to reduce the expression levels of p34 and cofilin individually or together (Fig. 5). When transfected into cells, the siRNAs nearly abolished the expression of the ap-

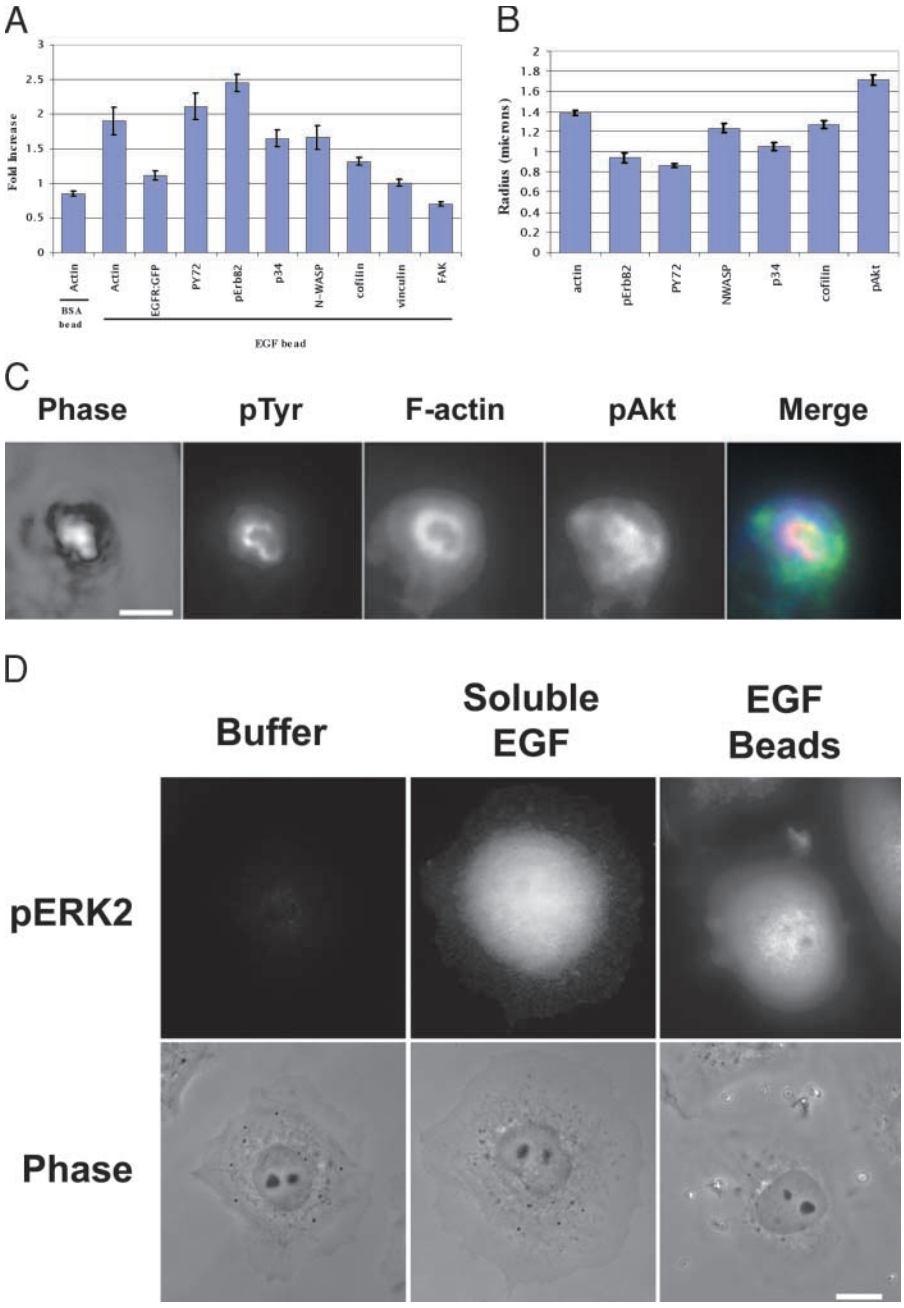

Figure 3. **Localization of signaling proteins around EGF beads.** (A) The relative increase in signal for the indicated molecule near the bead (5 min after application), relative to plasma membranes far from the bead in the same plane of focus, is given. Data are mean ± SEM from >33 cells in two or more separate experiments. (B) The areas of increased intensity of the samples measured in A were determined, and a radius calculated, assuming a circular shape. Means and SEM from >33 cells in two or more experiments are shown. (C) Comparison of phase, phosphotyrosine (pTyr, red), F-actin (blue), and phosphoAkt (pAkt, green) distributions in one cell stimulated with an EGF bead. Bar, 2 μm. (D) Global ERK activation. Cells were stimulated with buffer (left), 10 nM soluble EGF (middle), or EGF beads (right) for 5 min and then fixed and stained for phosphoERK (fluorescence on top, phase images on bottom). Beads are phase bright in the right hand phase image. Bar, 10 μm.

propriate proteins whereas control siRNA had no effect. Knock-down of either p34 or cofilin individually decreased the protrusion response and the total positive response by half. When both constructs were expressed in the same cell line, the protrusion response was practically ablated, and total positive response dropped by ~80%. Therefore, both Arp2/3 complex and cofilin can mediate localized actin polymerization responses.

In this report, we show that localized activation of the EGFR induced localized actin polymerization independent of receptor density. This activation was dependent on the kinase activity of the EGFR and actin polymerization. Consistent with the localized actin polymerization, proteins associated with the regulation of actin polymerization at the leading edge of lamellipodia were also localized to the membrane where the EGF bead was bound. Unlike experiments using fibronectin-coated beads (Miyamoto et al., 1995), proteins specific to focal adhesions did not accumulate near the EGF bead, indicating that this is not an adhesion-based response. PI3-kinase and rho family GTPases were not necessary for the localized actin polymerization; however, either p34 or cofilin was required.

The localized actin polymerization response described here resolves a possible paradox posed by the evidence for global activation of the EGF receptor (Verveer et al., 2000; Sawano et al., 2002): how cells that overexpress the EGF receptor still display chemotaxis to EGF if signaling from the EGFR is global. We propose that a key cytoskeletal response involved in cell motility, actin polymerization, remains local even in cells overexpressing the EGFR. We have evaluated

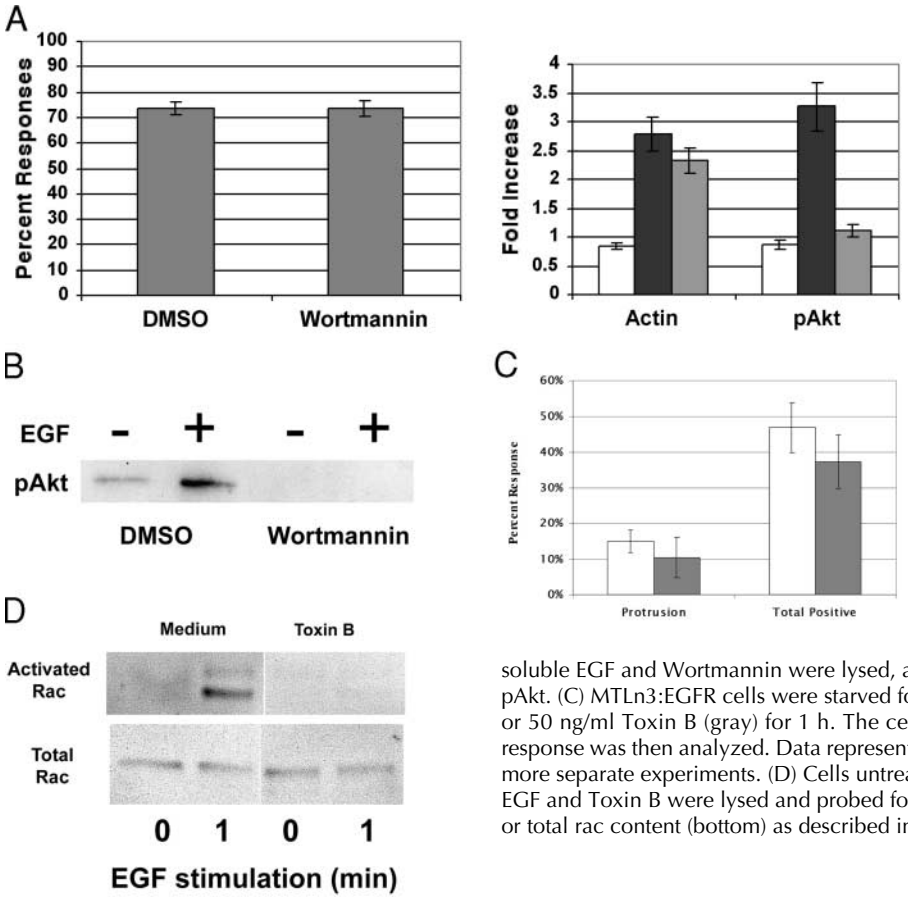

Figure 4. **The EGF bead response is PI3-kinase and rho family GTPase independent.** (A) MTLn3:EGFR cells were starved for 1 h, and then treated with DMSO or 100 nM Wortmannin for 15 min. Cells recovered for 1 h, before being treated with either EGF beads for 5 min (A) or 10 nM soluble EGF (B) for 3 min. (A, left) The percentage of cells responding to beads treated with Wortmannin or DMSO. Data represent the mean ± SEM from >120 cells in three separate experiments. (A, right) Wortmannin effects on actin polymerization and phosphoAkt near beads. BSA beads (white), or EGF beads (black and gray), were given to cells that were pretreated with DMSO (white, black) or 100 nM Wortmannin (gray). The relative increase in staining for F-actin and pAkt near the beads compared with plasma membranes far from the beads was measured. Data represent the mean ± SEM from >60 cells in two or more separate experiments. (B) Cells untreated or treated with 10 nM soluble EGF and Wortmannin were lysed, and the whole cell lysates were probed for pAkt. (C) MTLn3:EGFR cells were starved for 1 h, and then treated with DMSO (white) or 50 ng/ml Toxin B (gray) for 1 h. The cells were then stimulated for 80 s, and the response was then analyzed. Data represent the mean ± SEM from >120 cells in four or more separate experiments. (D) Cells untreated or treated for 1 min with 5 nM soluble EGF and Toxin B were lysed and probed for activated rac by GST-CRIB pulldown (top) or total rac content (bottom) as described in Materials and methods.

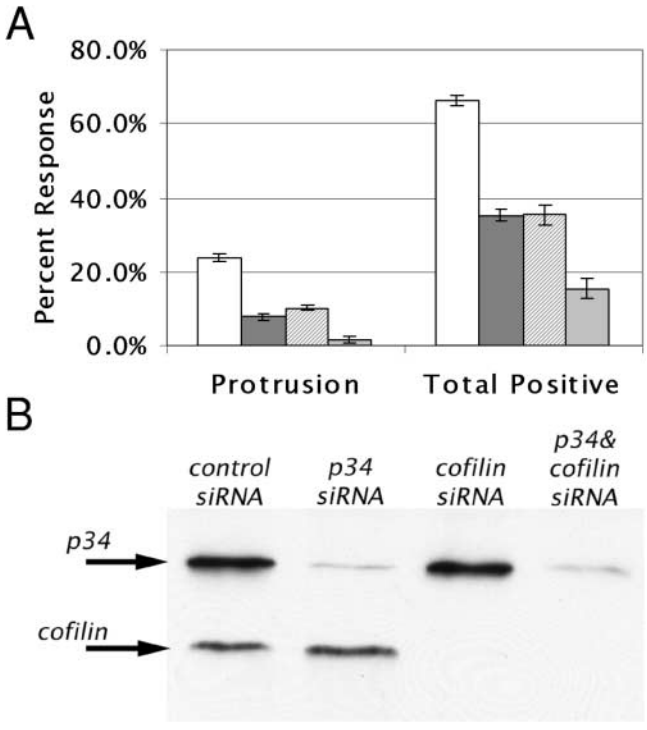

Figure 5. **The Arp2/3 complex and cofilin work synergistically to create the EGF bead response.** (A) MTLn3:EGFR cells transfected with control (white), p34 (dark gray), cofilin (stripes), or both p34 and cofilin siRNA (light gray) for 4 h. Cells were then cultured for 24–48 h before being analyzed. Cells were stimulated with EGF-coated beads for 80 s, fixed, and stained for rhodamine phalloidin, and the cell response was analyzed. Data represent the mean ± SEM from >180 cells in three separate experiments. (B) Lysates from cells treated with siRNA as in A were blotted for cofilin and p34 protein expression levels.

two signaling pathways activated by the EGF receptor (ERK activation and actin polymerization), and it will be important in future studies to evaluate the spatial spread of other signaling pathways emanating from the EGF receptor. An attractive general model for chemotactic orientation proposes a comparison occurring between a short-range, localized positive signal and a longer-range, negative signal that reflects global receptor activation. ERK activation could represent a global, negative signal downstream of EGF receptor activation, which may be important in deadhesion. One possible mechanism by which ERK could act as a negative signal is through induction of deadhesion by activating calpain, a protease that prefers to digest focal adhesion–associated proteins (Glading et al., 2000). The combination of the local actin polymerization we report here with global signals (such as activated ERK and the global activation responses [Verveer et al., 2000; Sawano et al., 2002]) could contribute to the gradient discrimination mechanisms that enable chemotactic responses. For a cell in a spatial gradient of soluble chemoattractant, the likelihood of a specific region of the cell extending or retracting would be dependent on the weighted sum of positive signals (such as actin polymerization) and negative signals (possibly activated ERK): a region will be affected by each receptor based on the recep-

tor's distance from the region and the receptor's activation level due to ligand binding. Models of amoeboid chemotaxis will be constrained to propagation distances from the receptor of ~1 μm for actin polymerization and at least 10 μm for activated ERK.

Materials and methods

Cells and reagents

MTC, MTLn3, and MTLn3:EGFR-GFP cell lines were described previously (Neri et al., 1982; Bailly et al., 2000). MCF7-EGFR, MDA-MB-435-EGFR, and MTLn3-EGFR cells were generated using an EGFR–PLXSN construct provided by David Stern (Yale University) and confirmed using FACS and ELISA. MTLn3-PLXSN cells were empty vector controls. Cells were maintained in αMEM with 5% FCS and antibiotics (Segall et al., 1996). For experiments, cells were plated on MatTek dishes in complete media overnight. Typically, cells were starved for 3 h in L15 media (GIBCO BRL) supplemented with 0.35% BSA. Cells were then stimulated with murine EGF (GIBCO BRL), EGF-bound beads, or BSA-coated beads , or left untreated. Live imaging was performed as described (Segall et al., 1996).

Biotin-EGF (Molecular Probes) was bound to strepavidin-bound magnetic beads (Pierce Chemical Co.) in PBS at room temperature for 1 h and was then washed 5 times to remove unbound EGF. BSA-coated beads were prepared by incubating the strepavidin beads in BSA. Beads were stored at 4°C in sodium azide and washed once in L15-BSA before use.

Rhodamine phalloidin was obtained from Molecular Probes. Rabbit anti-p34 and anti-cofilin antibodies were described previously (Chan et al., 2000; Bailly et al., 2001). The following antibodies for immunofluorescence were used: PY72, anti-phosphotyrosine (BabCO), goat anti–N-WASP (Santa Cruz Biotechnology, Inc.), anti-phosphoAkt (Ser473) antibody (Cell Signaling), anti-phosphoErbB2/HER-2 (Y1248) (Upstate Biotechnology), mouse monoclonal anti-vinculin (Sigma-Aldrich), and mouse monoclonal anti-FAK (Transduction Laboratories). Secondary antibodies, anti–mouse Cy5 and FITC, anti–rabbit Cy5, and anti–goat FITC, were from Jackson ImmunoResearch Laboratories. The following inhibitors were used: PD153035 and wortmannin (Calbiochem), cytochalasin D and nocodazole (Sigma-Aldrich), and *C. Difficile* toxin B (List Biological Laboratories).

Fluorescence microscopy and image analysis

Cells were fixed in 3.7% formaldehyde in fix buffer for 5 min at 37°C, treated at room temperature with 0.5% Triton X-100 for 10 min in fix buffer, and then 1 g/ml glycine for 10 min in fix buffer (Bailly et al., 2001). The cells were washed in TBS five times. F-actin structures labeled with rhodamine phalloidin for 20 min (Molecular Probes). Primary antibodies were incubated for 1 h at room temperature (except phosphoAkt and phosphoERK were overnight at 4°C), followed by secondary antibody for 45 min at room temperature and mounted. Cofilin immunofluorescence was performed as described (Eddy et al., 2000) with fixation time of 1 h.

The mean pixel intensity at the site of the bead was obtained by tracing the area immediately around the bead. In the same cell, the mean pixel intensity of the actin cortex under membranes, which were in focus but not associated with a bead, was obtained for comparison. The fold increase indicates the mean pixel intensity at the site of the bead over the mean pixel intensity of the actin cortex not in contact with a bead.

Western blot analysis

Cells were lysed in buffer containing 50 mM Tris, 150 mM NaCl, 0.5 mM EDTA, 10 mM NaI, 1% NP-40, 0.1% Triton, 1 mM $NaVO_3$, 2 mM PMSF, 10 μg/ml leupeptin, and 2 U/ml aprotinin, pH 7.4. Equal amounts of protein were run and blotted onto nitrocellulose. Primary antibodies were used at 0.1 mg/ml (anti-pAkt; Cell Signaling), anti-p34 (Upstate Biotechnology), anti-cofilin (from John Condeelis, Albert Einstein College of Medicine, Bronx, NY). HRP-conjugated anti–rabbit antibody (Amersham Biosciences) was then added followed by detection of antibody with ECL (Amersham Biosciences).

Rac activation assay

pGEX-2T human PAK1 GTPase-binding domain (hPAK 67–150) was expressed in *Escherichia coli* as a fusion protein and bound to glutathione–Sepharose beads. EGFR-overexpressing MTLn3 cells were treated with 40ng/ml Tox B *C. Dificile* Toxin B for 90 min at 37°C then stimulated with 5 nM EGF or buffer for 1 min. The cells were washed with cold PBS containing 1 mM sodium vanadate and lysed in MLB lysis buffer (25 mM Hepes, pH 7.5, 1% Igapal, 150 mM NaCl, 10 mM $MgCl_2$, 10% glycerol, 1 mM EDTA, 1 mM vanadate) with protease inhibitors (1 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml aprotinin). 8 μg of GST-PAK-sepharose beads was incubated in cell lysates at 4°C for 30 min, washed three times with MLB lysis buffer, and suspended in 50 μl Laemmli sample buffer. Proteins were separated by 14% SDS-PAGE, transferred to PVDF membrane, and blotted with mononclonal anti-Rac antibody (23A8; Upstate Biotechnology).

Small interfering RNA

Control, nonsilencing siRNA (AATTCTCCGAACGTGTCACGT), cofilin (AAGGTGTTCAATGACATGAAA) (Ghosh, M., G. Mouneimne, M. Sidani, and J. Condeelis, personal communication) and p34 siRNA (AAGGAACTTCAGGCACACGGA) were purchased from QIAGEN. Cells were transfected with 100 nM siRNA using oligofectamine (Invitrogen) 24 or 48 h before use.

Online supplemental material

Video 1 shows a phase–contrast movie of MTLn3:EGFR cells exposed to EGF beads at frame 12, one frame per 15 s. Video 2 shows a three-dimensional projection series from a confocal z-series of a cell showing localized actin polymerization (red) around an EGF bead (green). Video 3 shows a three-dimensional projection series from a confocal z-series of a cell showing an actin protrusion induced by an EGF bead (green). All supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200303144/DC1.

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