

# The Role of Ral A in Epidermal Growth Factor Receptor-regulated Cell Motility<sup>1</sup>

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## Abstract

**Tumor cell motility is one of the rate-limiting steps of invasion, which defines progression toward a more malignant phenotype. Elevated expression of epidermal growth factor (EGF) receptor in many cancers is associated with progression of superficial to invasive forms of the disease and is sometimes found in tumors that also have activating Ras mutations, suggesting that both events contribute to tumor invasion. Here we show that EGF stimulates motility in human tumor cell lines, which harbor activating Ha-RasV12 via a novel signal transduction pathway mediated by the small GTP-binding proteins RalA and RhoA but independent of Rac1 and Cdc42. On EGF stimulation, RalA localizes to the cell membrane. In addition, activation of RalA and expression of Rho were increased by EGF stimulation in both the nonmetastatic and metastatic variants of the same cell line. However, elevated levels of constitutively activated RalA were only found in the metastatic variant. This is the first demonstration of an essential role for Ral in EGF-mediated cell motility and its potential contribution to tumor metastasis in human cancer.**

## Introduction

EGFR<sup>3</sup> expression level in bladder cancer is associated with higher rates of progression from superficial to invasive forms of the disease (1). In addition, elevated expression of EGFR is sometimes found in tumors that also harbor activating Ras mutations (2), suggesting that EGF signaling and Ras activation are not redundant events but instead that each uniquely contributes to tumor progression. Hence, understanding the EGFR-mediated signaling pathways leading to bladder tumor invasion would help in the future rational design of therapeutic strategies for its inhibition. Because bladder cancer invasion is a complex multistep cascade, we sought to focus our studies on determining the impact that EGFR expression has on one of the rate-limiting steps necessary for invasion, namely cell motility, a process composed of either chemokinesis and/or chemotaxis. In the present study, we elucidate the pathways that regulate chemokinesis in the nontumorigenic T24 human bladder cancer cell line and have compared key components of this pathway in a tumorigenic and metastatic variant, T24T (3, 4). Whereas both cell lines have an activating Ha-RasV12 mutation, their motility can be further stimulated by EGF. Here we show that EGF mediates motility via a novel signal transduction pathway mediated by the small GTP-binding proteins RhoA

and RalA and that elevated levels of constitutively activated RalA are only found in the metastatic bladder cancer cell lines. This is the first demonstration of an essential role for Ral in EGF-mediated cell motility and its potential contribution to tumor metastasis in human cancer allowing the potential future rational therapies targeted at the inhibition of tumor invasion and metastasis.

## Materials and Methods

**Cell Lines, Chemokinesis Wound Assay, and Confocal Image Acquisition.** T24 and T24T human bladder cancer cell lines with nonmetastatic and metastatic phenotypes, respectively, and their growth conditions and doubling time of 30 h have been described previously (3, 4). EGF-induced migration was assessed by measuring the movement of cells into a scraped, acellular area or “wound assay” as described (5). For the pharmacological inhibitor assays, cells were plated in DMEM-F12 media plus 5% serum and grown to ~90% confluent, serum starved for 24 h in HyQ-CCM1 media (Hyclone, Houston, TX), scraped with a pipette tip, and assayed over a 12-h time period with and without EGF. A time-0 control was taken by fixing wells immediately after scraping. The assay was terminated when the EGF-stimulated control wound was completely closed, which occurred at ~12 h. The cells were then fixed in 100% methanol and stained with crystal violet. Analysis of the wounds was performed by digitally capturing the wounded area using an Olympus Stereo dissecting scope as described (3, 4). The wound area closure was quantified using Image-Pro Plus (Media Cybernetics, Silver Spring, MD) software. The threshold values for each wound were changed until the acellular area appeared completely white and the cell-occupied areas appeared completely black. The percentage of wound closure was calculated by dividing the area occupied by cells in the EGF-stimulated well by the area of the unstimulated well. For confocal assays, live cells were imaged on an Olympus Fluoview Dual Channel Confocal microscope using a 40× water immersion lens. For the RalDN construct, a Z-axis stack of images in 1 μm steps was summed using the Fluoview software package.

**Pharmacological Inhibitors and Growth Factors.** Murine epidermal growth factor, PMA, neomycin sulfate, Wm, SS, T-But, N-But, MitoC, and Fbn were purchased from Sigma Chemical Co. U73 and Ly were purchased from Biomol. C56, AG1478, 18-0-CH3, D609 (6), BIM, TG, BFA, and Prop were purchased from Calbiochem. All pharmacological inhibitors and growth factors were reconstituted in the carrier recommended by the manufacturer. None of the carriers at the concentrations used had any effects on the wound assay (data not shown). All pharmacological inhibitors were evaluated at three different concentrations (0.5 IC<sub>50</sub>, IC<sub>50</sub>, and 2 × IC<sub>50</sub>) using the published IC<sub>50</sub> for human cancer cell lines in the literature to evaluate specificity and toxicity.

**Plasmid Constructs and Transfection.** The WT Rlf construct, pMT2-HA-Rlf-WT, DN Rlf construct, pMT2-HA-Rlf-deltaCAT-CAAX, WT Ral construct, pMT2-HA-Ral WT, and DN Ral construct, pMT2-HA-RalN28, have been described elsewhere (7), as were the Rac1 (8), RhoA (9), and Cdc42 (10) constructs, pUM-Rac1-WT, pUM-Rac1-N17, pUM-RhoA-WT, pUM-RhoA-N19, pCDNIIIIB-cdc42-WT, and pCDNIIIIB-cdc42-N17. The WT and DN Ral-EGFP fusion constructs were generated by PCR amplifying WT Ral from the pMT2-HA-Ral WT and DN Ral from the pMT2-HA-RalN28 constructs described above, adding *Hind*III and *Bam*HI sites to the NH<sub>2</sub>- and COOH-terminal primers and directly subcloning the PCR product, cut with *Hind*III and *Bam*HI into *Hind*III- and *Bam*HI-cut pEGFP-C3 (Clontech, Palo Alto, CA). Transfections were performed by lipofectin (Life Technologies, Inc.) using the manufacturer’s instructions. Rapid selection of cells containing transfected plasmid constructs was achieved by mixing a 5:1 ratio of expression construct to selection plasmid, pBabe, which contains a puromycin selectable marker driven by the thymidine kinase promoter. One day after

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<sup>3</sup> The abbreviations used are: EGFR, epidermal growth factor receptor; PMA, phorbol 12-myristate 13-acetate; Wm, Wortmannin; SS, staurosporine; T-But, Tert-butanol; N-But, 1-Butanol; MitoC, Mitomycin C; U73, U73122; Ly, LY294002; C56, Compound 56; AG1478, Tyrosinase inhibitor AG1478; D609, Tricyclodecan-9-yl-xanthate; BIM, bis-indolyl-maleimide I; TG, Thapsigargin; BFA, Brefeldin A; Prop, propanolol; WT, wild type; DN, dominant negative; Total, total protein; PLC, phospholipase C; PI, phosphatidylinositol; PI3K, phosphatidylinositol 3-kinase; DAG, diacylglycerol; PKC, protein kinase C; PLD, phospholipase D; PA, phosphatidic acid; Arf, ADP ribosylation factor; GFP, green fluorescent protein; EGFP, enhanced green fluorescent protein; PAK, p21 activated kinase 1.

transfection and recovery, 1  $\mu\text{g/ml}$  puromycin (Sigma Chemical Co.) was added to the cultures overnight. The mock-transfected cells were completely dead during the same rapid selection period.

**Ral, Rac, and Cdc42 Activation Assays and Rho ABC Western.** The endogenous RalA, Rac1, and Cdc42 activation assay was performed as suggested by the manufacturer (Upstate Biotech, Lake Placid, NY). The amount of RalA protein bound to the RalBP1-agarose beads was quantitated by horseradish peroxidase chemiluminescence as described below. In the case of Rac1 and Cdc42 activation assays, a nonhydrolyzable GTP analogue GTP $\gamma\text{S}$  was added to the uninduced sample to assay the total amount of Rac1 and Cdc42 capable of binding in this assay. Total and affinity-purified protein samples were Western blotted, and proteins were detected by specific antibodies (Cytoskeleton, Denver, CO) followed by chemiluminescence Western blot using an Alpha Inotech (San Leandro, CA) gel documentation system and Supersignal West fempto reagent (Pierce, Rockford, IL). Western blot for total RhoA, RhoB, and RhoC isoforms was performed according to the manufacturer's protocol (Upstate Biotech). Equal loading was confirmed by Ponceau S staining of transferred protein on the polyvinylidene difluoride membrane, imaged by scanning.

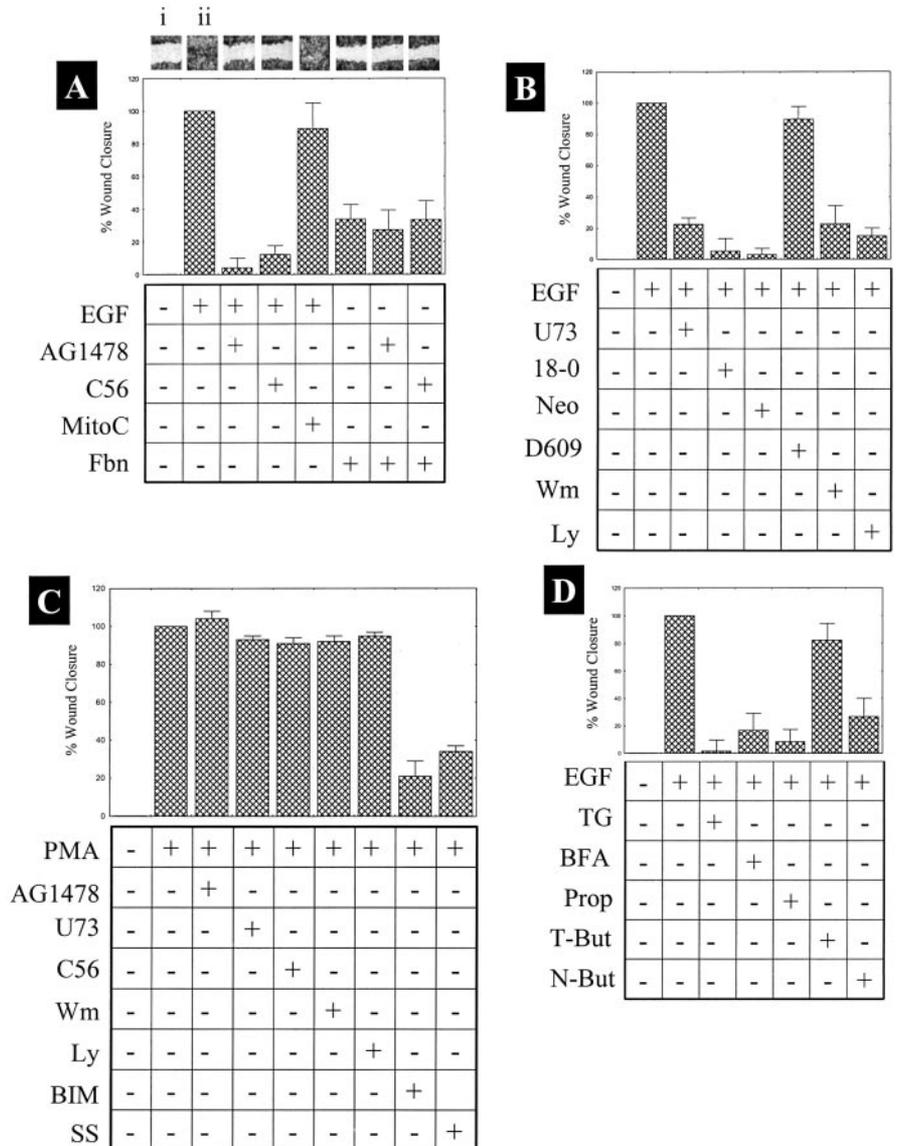
**Results and Discussion**

In the wound assay, T24 chemokinesis was stimulated by EGF. Time-course and dose-response experiments (data not shown) deter-

mined optimal conditions leading to wound closure in response to EGF (Fig. 1A, *i* and *ii*). This was specific and dependent on EGFR activity. C56 and AG1478, selective inhibitors of EGFR tyrosine kinase activity (11), completely inhibited EGF-induced wound closure while not affecting chemokinesis in response to alternative stimuli, such as Fbn (Ref. 12; Fig. 1A). Closure was not attributable to proliferation at the edge of the wound, because the addition of MitoC, commonly used to exclude this possibility (13), did not have any effect on EGF-induced wound closure (Fig. 1A).

Pharmacological inhibitors were used to define the EGFR-mediated pathways responsible for stimulation of chemokinesis. Proteins known to be phosphorylated after EGFR tyrosine kinase activation include PLC $\gamma$ , a PI-specific phospholipase, and PI3K. Using PLC-selective (6) inhibitors D609, U73, and 18-0-CH<sub>3</sub>, we show that a PI-PLC but not phosphatidylcholine-PLC is necessary for the EGF effect (Fig. 1B). PI3K activity was also necessary for EGFR motility signaling as determined by inhibition with Wm and Ly. Thus, PI-PLC and PI3K are downstream effectors of EGFR-mediated chemokinesis in the presence of a mutated RasV12. Because DAG is produced by PLC enzymatic activity (6), we determined the involvement and hierarchical position of DAG and its most well-characterized effector,

Fig. 1. Signaling intermediates affecting the chemotaxis of T24 cells in the wound assay. Control cells were incubated in serum-free media (*i*), and the assay was terminated when the EGF-stimulated control wound was completely closed, which occurred at  $\sim 12$  h. (*ii*). A, chemotaxis of T24 cells in response to EGF and Fbn. Top panel, a typical field used to quantitate motility and generate the graphs below. The final concentration of reagents in the assay media was EGF, 10 ng/ml; AG1478, 1  $\mu\text{M}$ ; C56, 10 nM; MitoC, 0.1  $\mu\text{g/ml}$ ; and Fbn, 5  $\mu\text{g/ml}$ . B, EGF-stimulated chemotaxis of T24 cells in the presence of pharmacological inhibitors of EGFR. Measurement of chemotactic response in the presence of EGF (10 ng/ml) and inhibitors was carried out as in A. Final concentration of the inhibitors present in the assay was U73, 5  $\mu\text{M}$ ; 18-0, 5  $\mu\text{M}$ ; neomycin sulfate, 50  $\mu\text{M}$ ; D609, 20  $\mu\text{M}$ ; Wm, 1  $\mu\text{M}$ ; and Ly, 30  $\mu\text{M}$ . C, T24 cell chemotaxis in response to PMA and pharmacological inhibitors of DAG or EGFR signaling pathways. Chemotaxis in the presence of 100 nM PMA was measured as in A. Final concentration of inhibitors was AG1478, 1  $\mu\text{M}$ ; U73, 5  $\mu\text{M}$ ; C56, 10 nM; Wm, 1  $\mu\text{M}$ ; Ly, 30  $\mu\text{M}$ ; BIM, 1  $\mu\text{M}$ ; and SS, 0.1  $\mu\text{M}$ . D, chemotaxis of T24 cells in response to EGF and inhibitors of PLD1 or Arf. Wound assays as in A were conducted in the presence of 10 ng/ml EGF and the following inhibitors: TG, 1  $\mu\text{M}$ ; BFA, 50 ng/ml; Prop, 50  $\mu\text{M}$ ; T-But, 2%; and N-But, 2%.



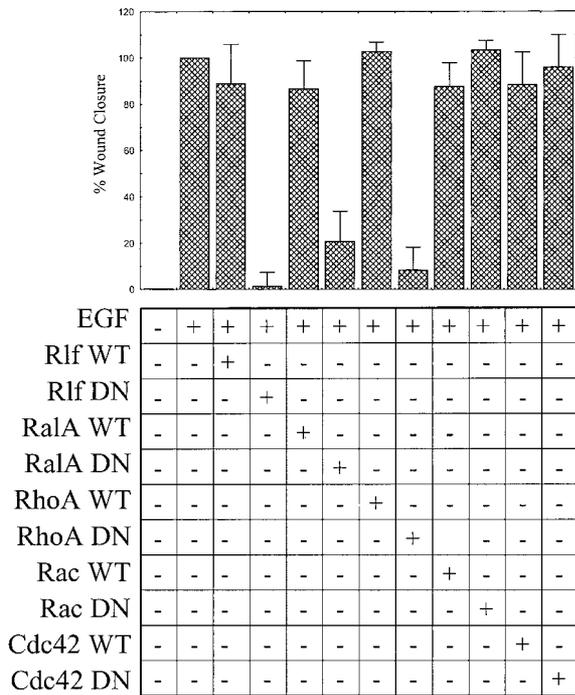


Fig. 2. Chemotactic response to EGF by T24 cells transfected with expression constructs for small GTPases. T24 cells were transfected with either empty vector (EGF + control), WT, or a DN form of the gene for the small GTPases RalA (7), RhoA (9), Rac1 (8), Cdc42 (10), or Rif (7), followed by rapid puromycin selection. Wounding and relative migration into a wound were quantitated as in Fig. 1A.

PKC, in EGF signaling, leading to chemokinesis. PMA, a DAG analogue, had similar effects to EGF stimulation in the wound assay, and its effect was not inhibited by AG1478, U73, or Ly (Fig. 1, B and C), suggesting that DAG lies downstream of EGFR, PI-PLC, and PI3K in this signaling cascade. In contrast, SS, a potent but nonselective protein kinase inhibitor, and BIM, an SS derivative that has a 350-fold selectivity for PKC over cyclic AMP-dependent protein kinase (14), inhibited both PMA- and EGF-induced chemokinesis. Therefore, activation of PKC is a downstream effector of EGF, PI3K, PLC, and DAG in this pathway.

A known target of PKC activation is PLD1. PLD1 removes the choline moiety from phosphatidylcholine, producing PA. PLD activation by PKC and Arf 1 has been shown to be critical for vesicular transport and cell motility in other systems (15). Inhibition of Arf GDP-GTP exchange by BFA (16) prevented chemokinesis induced by EGF. Biochemical inhibition of PA production by PLD using N-But alcohol prevented wound closure, but the inactive isomer T-But (17) did not (Fig. 1D). Prop, an inhibitor of phosphatidate phosphohydrolyase activity in intact cells, was in turn used to block the production of DAG from PA (17), and this also inhibited EGF-induced chemokinesis. Taken together with the results obtained from the SS and BIM experiments above, we conclude that DAG levels are provided by two sources (resulting from PLD and PLC activity) in this system, both of which are necessary for EGF-mediated motility. In addition, depletion of intracellular calcium stores by prolonged TG treatment inhibited EGF-stimulated motility (Fig. 1D). This is significant because calcium activates PKC, and Ras-independent activation of the monomeric G protein RalA has been shown recently to require calcium (18). Because PLD can also be activated by Rac1, Cdc42, RhoA, and RalA (17), we transfected T24 cells with expression constructs for DN and WT forms of these molecules and with Rif, a Ral guanine nucleotide exchange factor (19). Cell lines harboring the constructs were obtained by rapid puromycin selection as described and used in

the wound assay (Fig. 2). Both RalA DN and Rif DN transfection blocked EGF-induced chemokinesis, demonstrating a novel role for Ral as a key player in EGF-mediated chemokinesis. RhoA DN was the only member of the Rho family that inhibited motility.

To further implicate Ral activation in EGF-induced chemokinesis, we constructed vectors that express either a WT or DN RalA, fused to GFP. These constructs were then transfected into T24 cells, which were treated with EGF. Using confocal scanning laser microscopy, we found that EGF induces a shift in localization of the RalA-GFP fusion protein from the cytoplasm to the membrane (Fig. 3, A and B) in T24 cells. Interestingly, DN Ral-GFP fusion protein was localized to the entire circumference of the membrane (Fig. 3C) and caused these cells to round up and increase in height (data not shown). In contrast to the more aggressive T24T cells, Ral-GFP fusion protein was localized to the membrane without the need of EGF stimulation (Fig. 3D). These results suggest that Ral is a key mediator of cellular chemokinesis.

The lack of involvement of Rac1 and Cdc42 in EGF-induced chemokinesis in our system is surprising because RalBP1, a specific GTP-bound RalA effector, is a GTPase-activating protein for Rac1 and Cdc42 (20). Thus, we confirmed that RalA is being activated by EGF and PMA and that this activated RalA is capable of binding RalBP1 (Fig. 4A). No increase in total RalA expression was observed. This result clearly demonstrates that there is an EGF-mediated pathway that can augment RalA activation above levels induced by activated Ras. Significantly, the levels of constitutively activated RalA in the absence of EGF or PMA were higher in the more aggressive T24T cell line, and RalA activation was stimulated by EGF and PMA in both cell lines, despite the activating RasV12 mutation.

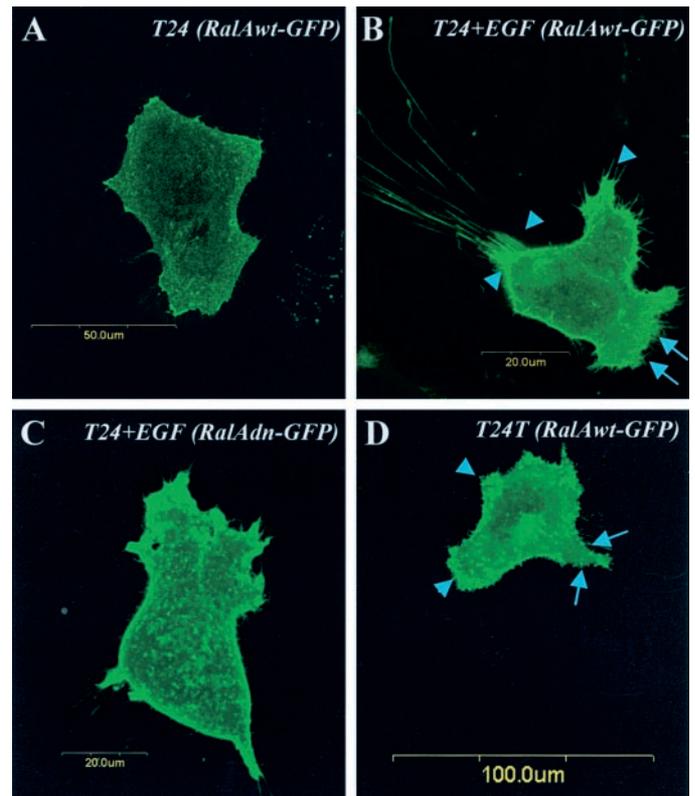


Fig. 3. Cellular translocation of Ral during EGF-induced chemokinesis in T24 and T24T. RalA WT-GFP (A and B) and RalA DN-GFP (C) fusion constructs were transiently transfected into T24 cells and serum starved, and EGF was either not added (A) or added (B and C) at 10 ng/ml and incubated for 2 h. Cells were imaged using confocal microscopy as described. RalA WT-GFP (D) was transiently transfected into T24T cells, which were then serum starved for 2 h. Arrowheads, region of Ral localization; arrows, region devoid of membrane-bound Ral.

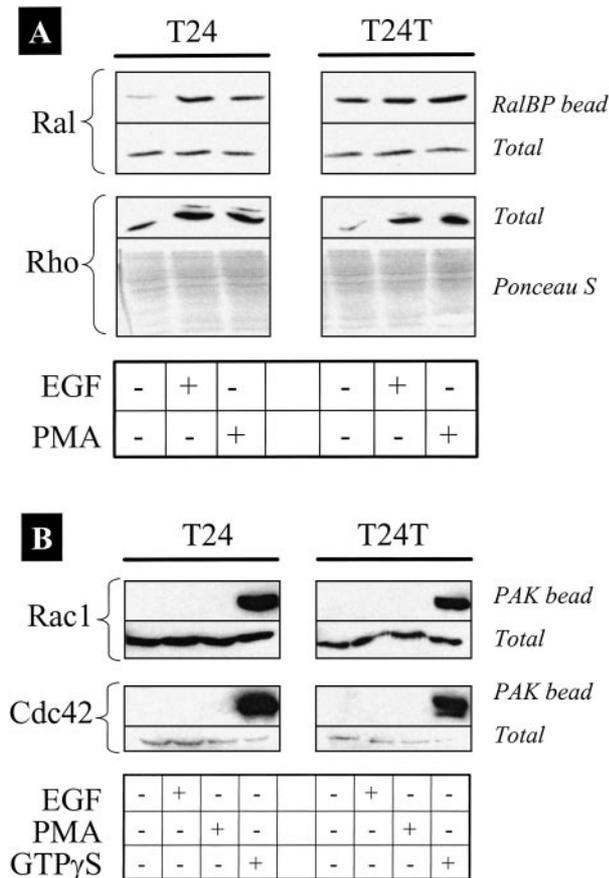


Fig. 4. Activation of small GTPases in EGF- or PMA-stimulated nonmetastatic and metastatic cancer cells. *A*, activation of RalA and expression of Rho in EGF- or PMA-stimulated T24 and T24T cells. Endogenous RalA activation assay was performed as described on cultures of T24 and T24T (3, 4). Total and affinity-purified protein (*RalBP bead*) samples were Western blotted, and RalA protein was detected by a RalA-specific antibody followed by chemiluminescence. Before immunodetection with a RhoA, B, and C antibody, the polyvinylidene difluoride membrane was reversibly stained with Ponceau S to check equality of loading and transfer. *B*, activation of Rac1 and Cdc42 by EGF- or PMA-stimulated T24 and T24T cells. Endogenous Rac1 and Cdc42 activation assays were performed on the same samples as in *A*. Total and affinity-purified (*PAK bead*) protein samples were Western blotted, and Rac1 and Cdc42 proteins were detected by Rac1- and Cdc42-specific antibodies, followed by chemiluminescence as described.

Of the Rho family members, only Rho was perturbed by EGF or PMA. Rho protein expression increased by both treatments, and this increase was equivalent in T24 and T24T cells (Fig. 4A). Despite the presence of RasV12 in these cell lines, no activated Rac1 or Cdc42 was detected, as determined by binding to immobilized PAK (Fig. 4B). Furthermore, EGF and PMA stimulation did not result in Rac1 or Cdc2 activation. Addition of GTPγS to the cell lysates rescued Rac1 and Cdc42 binding to PAK, indicating that the assay worked and that these two proteins are capable of being activated.

In conclusion, we have elucidated a pathway that stimulates cell motility in response to EGF in cell lines harboring an activating Ras mutation. This observation implies that the EGF effect on motility cannot be attributed solely to Ras activation and may explain why some tumors exhibit both increased EGFR expression and Ras muta-

tion. We have identified key components of this pathway, including an essential role for RalA, a gene not associated previously with tumor cell motility. In addition, we have found that constitutive RalA activation is associated with tumor progression to a metastatic phenotype in a model of human cancer.

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