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The Rak tyrosine kinase associates with and internalizes the epidermal growth factor receptor

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Abstract

Src is the founding member of a diverse family of intracellular tyrosine kinases, and Src has a key role in promoting cancer growth, in part through its association with receptor tyrosine kinases. However, some Src-related proteins have widely divergent physiological roles, and these proteins include the Rak tyrosine kinase (also called Frk for Fyn-related kinase), which inhibits cancer cell growth and suppresses tumorigenesis. Rak phosphorylates and stabilizes the Pten tumor suppressor, protecting it from degradation, and Rak associates with the Rb tumor suppressor. However, the role of Rak in receptor-mediated signaling is largely unknown. Here, we demonstrate that Rak associates with EGFR (epidermal growth factor receptor), increasing in activity and EGFR binding after EGF stimulation, when it decreases the pool of EGFR present at the plasma membrane. EGFR-Rak binding is direct, requires the SH2 and SH3 domains of Rak for efficient complex formation and is not dependent on the Grb2 adaptor protein. EGFR mutations are associated with increased EGFR activity and tumorigenicity, and we found that Rak associates preferentially with an EGFR exon 19 mutant, EGFRΔ747-749/A750P, compared to wild-type EGFR. Furthermore, Rak inhibited mutant EGFR phosphorylation at an activating site and dramatically decreased the levels of EGFRΔ747-749/A750P from the plasma membrane. Together, the results suggest that Rak inhibits EGFR signaling in cancer cells and has elevated activity against EGFR exon 19 mutants.

Keywords: signaling, tyrosine kinase, Frk, EGFR, SH2, SH3

Introduction

Cancer progression is driven by a combined increase in proliferative signals and a decrease in tumor suppressor function. EGFR is one of the receptors driving tumor growth in a number of cancers (1), including lung cancer, and therapeutics targeting EGFR are a promising treatment approach for the disease (2). Current therapeutics include small molecule kinase inhibitors, such as erlotinib, and antibody fragments such as cetuximab (3-5). EGFR is mutated in a minority of cancer patients, and those with mutations in the ATP binding site have better response to EGFR small molecule inhibitors (6-8), while K-Ras mutations correspond with a poor response to the inhibitors.

Upon ligand stimulation, EGFR is internalized through a series of phosphorylation and ubiquitination reactions and either degraded or recycled to the plasma membrane (9). A number of intracellular sites of EGFR are phosphorylated during this process, and Y1173 is thought to contribute to EGFR internalization (10) and ERK activation (11), although in some cases Y1173 may also be important in receptor activation (12). EGFR also associates with Grb2, an SH3-SH2-SH3 (Src homology) adaptor protein (13), upon EGF stimulation (14). SH2 domains associate with phosphorylated tyrosine residues, while SH3 domains bind to proline-rich motifs (15). Many of the binding partners for EGFR during this process have been extensively characterized, but there are significant gaps in our knowledge of the trafficking and recycling of EGFR.

Rak (also called Frk for Fyn-related kinase) is a Src-related tyrosine kinase (16-19) containing SH2 and SH3 domains at its amino terminus and a carboxy-terminal tyrosine residue (20). Unlike many Src-related kinases, which have varying degrees of transforming activity (20), Rak is a putative tumor suppressor in breast cancer that arrests cell growth and suppresses tumorigenesis (21-23). The apparent mouse homologue of Rak, called Iyk (24), is lost in tumors (25) and inhibits cell growth in culture (26). In spite of its profound activity in cultured cells, mice lacking Iyk do not form tumors (27), suggesting that additional

tumorigenic events are necessary for Rak's tumor suppressor function to be detectable in an intact organism.

Rak binds and phosphorylates the tumor suppressor Pten (28), stabilizing Pten levels and triggering growth arrest (23). Many tumor types are deficient in Pten, suggesting that Rak may have other intracellular functions. Indeed, Rak binds to the retinoblastoma tumor suppressor Rb (21), although the biological consequences of this interaction are unclear. Rak also phosphorylates Src on the C-terminal tyrosine of Src (17), a negative regulatory site, suggesting that Rak may have broad effects on signaling.

Virtually nothing is known about the role of Rak in regulating receptor-mediated signaling. Src family kinases, which are structurally similar to Rak (17), bind to receptor tyrosine kinases and mediate receptor signaling. In the present study, we show that Rak phosphorylates EGFR on Y1173 and associates with EGFR in punctate intracellular sites, increasing the intracellular pool of EGFR. Furthermore, Rak preferentially associates with a mutant form of EGFR that is associated with tumor formation. The results suggest that Rak's tumor suppressive activities include the down regulation of receptor tyrosine kinases, including EGFR.

RESULTS

Rak alters EGFR Y1173 phosphorylation.

Rak is not readily expressed in many cancer cells and causes cell cycle arrest. For that reason, we utilized an adenovirus driving the expression of an epitope-tagged form of Rak (22) and infected A549 human lung cancer cells. With increasing doses of adenovirus, Rak was readily expressed (Figure 1A, first panel) and did not affect EGFR levels (Figure 1A, second panel), but suppressed EGFR-Y1068 phosphorylation (Figure 1A, third panel) and increased EGFR-Y1173 phosphorylation (Figure 1A, fourth panel). As for Y1068, EGFR-Y992 phosphorylation was progressively decreased with Rak expression (Supplemental

Figure 1). EGFR-Y1068 and Y992 are generally associated with activation of the receptor, while EGFR-Y1173 phosphorylation has been linked to receptor down-regulation (10, 11, 29).

Rak expression altered EGFR tyrosine phosphorylation in multiple cell lines. In NCI-H226 human squamous cell lung cancer cells, Rak expression (Figure 1B, top panel) did not affect EGFR levels (Figure 1B, second panel) and had only a minor effect on EGFR-Y1068 phosphorylation (Figure 1B, third panel) but increased EGFR-Y1173 phosphorylation (Figure 1B, fourth panel). We were concerned that adenoviral infection may have accounted for the change in phosphorylation, but an equal titer of a control adenovirus did not affect EGFR phosphorylation (Figure 1A and 1B, lane 1). Furthermore, transfection of Rak into HEK293 cells (Figure 1C, top panel) also decreased EGFR-Y1068 phosphorylation (Figure 1C, third panel) and increased EGFR-Y1173 phosphorylation (Figure 1C, fourth panel). Rak expression is minimal in many cancer derived cell lines, particularly lung cancer, but Rak expression is relatively abundant in Ovcar-3 ovarian cancer cells. RNAi targeting of Rak resulted in a decrease in EGFR-Y1173 phosphorylation after EGF stimulation (Figure 1D, lanes 3 and 4).

To determine whether Rak directly phosphorylated EGFR, or whether the increased phosphorylation occurred through a secondary partner, we tested Rak activity towards purified, recombinant EGFR 1141-1211 in an *in vitro* kinase assay. Because the EGFR fusion protein contains two tyrosine residues, phosphorylation was measured by western blot using an EGFR-pY1173-specific antibody, and Rak efficiently phosphorylated the fusion protein (Figure 1E, upper panel, lane 2). For the assays, Rak was immunoprecipitated (Figure 1E, middle panel) and incubated with purified fusion protein (Figure 1E, lower panel). The activity was not autophosphorylation from EGFR in the reactions, because precipitated EGFR did not phosphorylate the fusion protein (Figure 1F, upper panel, lane 2). We conclude that Rak increases EGFR-Y1173 phosphorylation, at least in part through direct phosphorylation of the receptor.

Rak co-precipitates and co-localizes with EGFR.

EGFR associates directly with Src family tyrosine kinases, and we found that EGFR precipitated in a complex with Rak (Figure 2A, top panel, lane 2) when Rak was immuno-precipitated from HEK293 cells (Figure 2A, second panel, lane 2). As a control, the same precipitation conditions in cells transfected with a control plasmid failed to precipitate either protein (Figure 2A, top two panels, lane 1). The level of EGFR in the lysates prior to the precipitations was unaffected (Figure 2A, third panel). The reverse experiment, immuno-precipitation of EGFR, yielded bands for both Rak (Figure 2B, top panel, lane 2) and EGFR (Figure 2B, lower panel, lane 2), while neither was present in control precipitation reactions (Figure 2B, lane 1). Unlike EGFR, precipitated Met tyrosine did not exist in a complex with Rak (Supplemental Figure 2A).

We then performed analogous experiments in A549 NSCLC cells, and again, EGFR precipitated with Rak (Figure 2C) and Rak precipitated with EGFR (Figure 2D). As above, the loading of each protein was equivalent in cell lysates. Rak is poorly expressed in tumor cells, perhaps because of its tumor suppressor function, and the above experiments relied on transfected Rak. However, Rak and EGFR are co-expressed in multiple cell lines, including SW480 human colon cancer cells, A431 human epitheloid cancer cells (Figure 2E) and HEK293 human embryonic kidney cells (Supplemental Figure 2B). In precipitation reactions of endogenous EGFR from each cell line, endogenous Rak was readily detectable (Figure 2F and Supplemental Figure 2C).

Rak decreases extracellular EGFR.

EGFR-Y1173 phosphorylation has been implicated in intracellular uptake of EGFR (10). To determine whether Rak affected the fraction of EGFR at the plasma membrane, we biotinylated the extracellular surface proteins of A549 cells infected with a control virus or an adenovirus driving Rak expression. Rak caused a decrease in EGFR levels on the

extracellular surface (Figure 3A, top panel, compare lanes 3 and 4). While the majority of Rak was intracellular (Figure 3A, second panel, lane 2), a fraction was labeled with biotin, suggesting a sub-population of the protein at the cell surface (Figure 3A, second panel, lane 4). PCNA served as a control for intracellular proteins and was enriched in the non-biotinylated fraction (Figure 3B). In contrast to EGFR, the levels of biotinylated Met were unaffected by Rak expression, suggesting a measure of specificity in the Rak-EGFR interaction (Figure 3C). The overall levels of membrane proteins were not markedly different, as judged by Coomassie staining of the fractionated proteins (Figure 3D). The EGFR analysis was repeated in triplicate and quantitation is shown in panel 3E, with the result being a 5-fold drop in extracellular EGFR. We obtained a similar result by transfecting Rak into A549 cells, although the extent of EGFR internalization was limited by the efficiency of the transfection procedure (Supplemental Figure 3).

To gain insights into the potential function of the Rak-EGFR complex, we determined the sub-cellular localization in which the proteins overlap. The localization of Rak is controversial, having been detected in the nucleus and cytoplasmic puncta (17, 21, 22). We expressed Rak in A549 NSCLC cells and detected Rak in a perinuclear region resembling the endoplasmic reticulum, in cytoplasmic puncta and to a lesser degree at the cell periphery (Figure 3F, lower center panel). In the absence of Rak, EGFR was readily detectable at the plasma membrane (Figure 3F, upper left panel, triangles), while EGFR localized to the perinuclear region when Rak was expressed (Figure 3F, lower left panel, triangles), where it co-localized with Rak (Figure 3F, lower right panel). The results suggest that Rak may associate with EGFR during the receptor's production, transport or processing.

The Rak SH2 and SH3 domains collaborate to increase the Rak-EGFR interaction.

Like other Src-related proteins, Rak has amino-terminal SH2 and SH3 domains and a carboxy-terminal regulatory tyrosine residue (17). We deleted the SH2 and SH3 domains,

separately or together, and tested co-precipitation with EGFR. Deletion of SH2 and SH3 domains increased Rak-EGFR binding by 5- and 3-fold, respectively (Supplemental Figure 4A, second panel, plotted in panel B). A western blot of the input lysates for the reactions is shown in the third and fourth panels. In contrast, deletion of both the SH2 and SH3 domains decreased EGFR binding by approximately 4-fold (Figure 4A, upper panel, lane 6, summary graph in Figure 4B), indicating that these domains collaborate to direct Rak-EGFR binding. While deletion of the amino-terminal domains decreased binding, a kinase-deficient mutant (K262R) or mutation of the carboxy-terminal Y497 had little effect on EGFR binding (Figure 4A, lanes 4 and 2, respectively). The kinase-deficient mutant also had no effect on EGFR-Y1173 phosphorylation, while wild-type Rak increased Y1173 phosphorylation (Supplemental Figure 4C).

EGFR is activated by ligand stimulation, and we determined the extent to which the Rak-EGFR complex changed upon stimulation. EGF increased the levels of Rak co-precipitating with EGFR by 3.3-fold within 5 minutes of treatment and remained elevated by 3.4-fold within 30 minutes (Figure 5A, top panels). There was also a 27% decrease in Rak expression after EGF stimulation (Figure 5A, lower panels). The results suggest that Rak-EGFR binding occurs at the highest levels during the internalization of the receptor. Because Rak phosphorylates EGFR, we then tested the extent to which Rak activity is altered by EGF stimulation. Five minutes after EGF stimulation, Rak activity increased by 2.8-fold (Figure 5B, lane 2) using an EGFR-Y1173-containing fusion protein as a substrate for an *in vitro* kinase assay.

One potential mediator of Rak-EGFR binding is the Grb2 adaptor protein, which is predicted to bind to Rak in protein interaction databases. Indeed, Grb2 co-precipitated (Figure 5C, third panel) with both Rak and EGFR (Figure 5C, top two panels, lane 2). In contrast, an inactivating Grb2-R86K mutation largely eliminated binding to Rak and EGFR (Figure 5C, top two panels, lane 3). We posited that Grb2 might act as an adaptor between

Rak and EGFR, but the levels of Rak co-precipitating with EGFR increased when the inactivating Grb2 mutant was co-expressed with the two proteins (Figure 2D, top panel, compare lanes 2 and 4), suggesting that the Rak-EGFR association is not dependent on Grb2. Because Grb2 binding was not essential for the Rak-EGFR interaction, we tested the possibility that Rak binds directly to EGFR. A GST fusion protein containing amino acids 1141-1121 of EGFR was incubated with Rak-transfected HEK293 cell lysates and analyzed by western blot. Rak was readily detected in an affinity precipitation with the EGFR fusion protein (Figure 5E, lane 2), while there was no binding of Rak to GST alone (Figure 5E, lane 1).

Rak binding is decreased in an EGFR mutant.

EGFR is capable of being activated by mutations, including the EGFR-Δ747-749/A750P mutation in exon 19 (6), which we will refer to as EGFR-Δ747. Wild-type and EGFR-Δ747 were expressed in MDA-MB-435 melanoma cells, which do not express EGFR (Figure 6A, first panel, lane 1), and the EGFR-Δ747 had an elevated level of tyrosine phosphorylation compared to the wild-type receptor (Figure 6A, second panel). Rak was co-expressed (Figure 6A, third panel), and EGFR-Δ747 co-precipitated with 4.4-fold higher levels of Rak than wild-type EGFR (Figure 6A, fifth panel and graph). To determine the effect of Rak on EGFR-Δ747 activation, cells were transfected with EGFR-Δ747, alone or in combination with Rak. Rak reduced EGFR-Y1068 phosphorylation by 4.8-fold (Figure 6B, compare lanes 2 and 3), and the effect was partially abrogated by EGF stimulation (Figure 6B, lanes 5 and 6).

Because Rak altered the membrane localization of wild-type EGFR, we considered that it may affect EGFR-Δ747. Wild-type or mutant EGFR were co-expressed with Rak, and cell surface proteins were monitored by biotinylation, as in Figure 3. The total levels of biotinylated proteins in the extract and of wild-type and mutant EGFR were equivalent in the

samples (Supplemental Figure 5A and B). Compared to wild-type EGFR, Rak expression dramatically decreased EGFR-Δ747 membrane levels (Figure 6C, lanes 5 and 6). EGFR-Δ747 was not detected in the unbiotinylated pool, except on very long blot exposures (Supplemental Figure 5C) possibly because activated EGFR frequently associates with a "triton-insoluble fraction" (30), and the extraction buffer for the membrane fractionation procedure contains 1% triton X-100 as detergent. Unlike A549 cells (Figure 3A, lower panel), Rak was not detectable at the plasma membrane in MDA-MB-435 cells (Figure 6C, second panel) and co-localized with PCNA inside the cell (Figure 6C, lower panel). In the absence of Rak, EGFR-Δ747 localized prominently to the plasma membrane (Figure 6D, upper panel, lane 5) until Rak was co-expressed (Figure 6D, upper panel, lane 6). We conclude that Rak preferentially associates with the EGFR-Δ747 mutant and potently down-regulates the mutant receptor.

Discussion

Rak is a Src-related tyrosine kinase that differs from other Src family members (20) in that it lacks an amino-terminal myristilation site and has growth inhibitory, rather than transforming, activity (21-23). Src-related kinases play an important role in multiple signaling pathways, including signaling by RTKs. Rak alters signaling by phosphorylating Pten on Y336, stabilizing Pten levels (23), but a direct role of Rak in receptor-mediated signaling is unknown. Here, we demonstrate that Rak precipitates with EGFR in cancer cells, phosphorylates EGFR directly, and alters the pool of EGFR at the plasma membrane.

Rak expression was very low in lung cancer cell lines (17), although Rak RNA is detectable in normal lung (17). Rak mutations have also been detected in lung cancer patients (31), although it is unclear whether heterozygous mutations alone can increase susceptibility to lung cancer. In addition to point mutations, Rak localizes to chromosome

6q21, a region that is commonly deleted in lung cancers (32). The Rak-related Iyk kinase is also frequently lost in cancers (25). In contrast, the related PTK6/BRK is present in the majority of tumors and has a more complex role in regulating cell growth (33), in that it inhibits growth of normal epithelial cells (34) but promotes growth of tumor cells, at least in part by phosphorylating $p130^{cas}$ (35), β-catenin (36) and Akt (37). PTK6/Brk also binds to EGFR in breast cancer cells, sustains EGFR activation and phosphorylates EGFR, but on Tyr845 (38), which is distinct from Rak. Potential interactions between Rak and PTK6/Brk are unknown and are the subject of ongoing investigation. In addition to EGFR, we also examined potential interactions between Rak and Met and did not detect a complex (Supplemental Figure 2). However, we cannot exclude the possibility that other receptor tyrosine kinases precipitate with Rak.

We found that individual deletion of the Rak SH2 or SH3 domains elevates levels of co-precipitating Rak and EGFR, which suggests that Rak does not bind to a single phosphorylated tyrosine or proline-rich sequence of EGFR. In contrast, deletion of both domains decreased EGFR binding, suggesting that the Rak SH2 and SH3 domains function in atypical manner for kinases related to the Src family. Indeed, Src family kinases are activated enzymatically by deletion of their amino-terminal domains, while Rak enzymatic activity is relatively unaffected (22). One possible explanation is that the Rak SH2 and SH3 domains act as a single entity for some substrates, rather than as individual modules.

We considered the model that the carboxy-terminal tyrosine of Rak might bind to an adaptor protein, bridging it to EGFR. However, Rak mutants deleted for the carboxy-terminal tyrosine bind normally to EGFR, which does not support the model. We then tested the hypothesis that Rak associates with EGFR via an EGFR-Grb2 complex (1). Indeed, Rak co-precipitates with wild-type Grb2, and the complex between Rak and Grb2 was absent when expressing a Grb2 mutant with an inactive SH2 domain (Figure 5), suggesting that the Grb2 SH2 domain is required for Rak binding. However, Rak lacks the consensus pY-X-N-X Grb2-

SH2 consensus sequence (39), suggesting that Grb2 may bind indirectly to Rak through an additional partner. Surprisingly, co-expression of Grb2, Rak and EGFR had little effect on the levels of the Rak-EGFR complex, and the dominant-negative Grb2 mutant elevated the Rak-EGFR association. We conclude that Rak-Grb2 binding inhibits Rak-EGFR binding, perhaps through competition between the Rak-Grb2 and Rak-EGFR complexes.

Our results support a model in which Rak-EGFR complexes increase after EGF stimulation. Rak activity is stimulated by EGF, and Rak phosphorylates EGFR directly on Y1173 in a biochemical assay. Phosphorylation of EGFR-Y1173 has been associated with increased EGFR uptake, and Rak hyper-expression decreased EGFR levels at the plasma membrane. In contrast, Rak decreased the phosphorylation of EGFR-Y1068, a binding site for Grb2 (40), along with Y1086 (41). EGF stimulation increased EGFR-Rak complex formation, Rak activity and EGFR-Y1173 phosphorylation.

EGFR mutations are frequently activating, drive tumor growth and increase susceptibility of tumors to EGFR inhibitors (8). The class I EGFR-Δ747-749/A750P mutant was isolated twice from erlotinib-responding lung cancer patients (6) and is transforming (42). The EGFR-Δ747-749/A750P mutant is closely related to the EGFR-Δ746-750 mutant (7), which exhibits increased downstream signaling and suppressed ubiquitination and internalization (43, 44). The regulation of EGFR by ligand binding is complex, involving phosphorylation, ubiquitination and binding of multiple proteins that usher EGFR to endosomes, where it is recycled to the plasma membrane or degraded (9). Our current model is that increased binding of Rak to the EGFR-Δ747-749/A750P mutant may increase endocytosis or slow recycling of the mutant protein. One prediction of this model is that Rak expression will be attenuated in tumors expressing mutant EGFR, and a test of this hypothesis is in progress.

The co-localization of Rak and wild-type EGFR in punctate intracellular sites (Figure 3) suggests that it may stabilize or delay the transit of an endosomal population of EGFR. These populations could include newly synthesized EGFR, in transit to the plasma membrane, or endocytosed EGFR. It is unclear whether EGFR exon 19 mutants are trafficked through the same pathways as the wild-type protein or whether Rak contributes to these pathways. However, our results suggest that Rak has the potential to attenuate signaling in tumor cells expressing EGFR exon 19 mutants. This may clinical implications in cancer patients expressing EGFR mutants and may potentially lead to therapeutics targeting the mutant EGFR receptors.

EXPERIMENTAL PROCEDURES

Cell lines and plasmids- A431, A549, BT549, H226, Ovcar-3 and SW-480 cells were obtained from the ATCC, cultured under the suggested conditions and verified by Genetica LLC (Cincinnati, OH). MDA-MB-435 melanoma cells were a generous gift from Dr. Rina Plattner and Sourik Ganguly. Cells were maintained in DMEM containing 10% serum supreme and antibiotics, except where described. The control and HA-Rak adenoviruses (based on the pShuttle-CMV plasmid) have been described previously (22). Rak siRNA was from Santa Cruz Biotechnology (Santa Cruz, CA) and was transfected using Oligofectamine (Invitrogen, Grand Island, NY). Purified epidermal growth factor was from Axcell Biosciences (Newtown, PA).

For the GST-EGFR fusion protein, a PCR product including the 3421-3633 sequence of the EGFR open reading frame was amplified using the primers EGFR+3421F-*Bam* (AATGGATCCACTGTCCAGCCCACCTG) and EGFR+3633R-*Xho* (AATCTCGAGTCATGCTCCAATAAATTCACTGCT), digested with *Bam*HI and *Xho*I and cloned into the comparable sites of pGEX-4T-1, forming the plasmid pLJ1. Rak was myc-tagged by sub-cloning into the *Xho*I and *Not*I sites of the plasmid pCMV-myc-N (BD

Clontech, Mountain View, CA) using the plasmid pcDNA3.1-Rak (21) as a template with Rak+1F-*Xho* (AATTCTCGAGGTATGAGCAACATCTGTCAGAGG) and Rak+1518R-*Not* (AATTGCGGCCGCTCATCTTATGAAGTTATTTGCATC) as primers, forming the plasmid pLJ3. Rak mutants lacking the single SH2 and SH3 domains were sub-cloned using the same approach from the plasmids pGFP-Rak-SH2Δ and pGFP-Rak-SH3Δ (22), forming the plasmids pLJ4 and pLJ5, respectively. The Rak-Y497F and Rak-K262R (KD) mutants were also sub-cloned similarly from the templates pGFP-Rak-Y497F and pGFP-Rak-KD (22), forming the plasmids pLJ7 and pLJ6. The Rak mutant lacking both SH2 and SH3 domains was constructed by amplifying the plasmid pLJ3 with the primers Rak+595F-Kpn (AATGGTACCGGCCTGTGTGTCAAGCTGGG) and Rak+138R-Kpn (AATGGTACCGTAGTGCCATGAATCTGTGA), forming the plasmid pLJ8. The pcDNA3.1-Rak-flag vector has been published previously (22).

The pcDNA3.1-EGFR vector was a gift from Drs. Penni Black of the University of Kentucky College of Pharmacy and William Pao of the Vanderbilt University Department of Medicine. The pBabe-EGFR-Δ747-749/A750P plasmid was purchased from Addgene (Cambridge, MA). Plasmids were transfected using the Transgin (APSBio, Gaithersburg, MD) reagent following the manufacturer's instructions.

Protein analysis- For immunoprecipitations, cells were lysed in 1% Triton Buffer (20 mM HEPES, pH 7.4, 50 mM KF, 50 mM β-glycerol phosphate, 150 mM NaCl, 2 mM EGTA, 1 mM sodium vanadate, 10% glycerol, 1% Triton X-100 and 1X protease inhibitor cocktail (Thermo Scientific, Rockford, IL) and precipitated with antibody and protein A/G-plus agarose (Santa Cruz Biotechnology, Santa Cruz, CA). Precipitated proteins were collected by centrifugation, washed five times with 1% Triton Buffer. For the first and third washes, pellets were washed with 1% Triton Buffer containing 500 mM NaCl. For affinity precipitations with a GST-EGFR fusion protein, 20 μg of GST fusion protein was incubated

with 2 mg of cell lysate in 1% Triton Buffer and incubated overnight. Centrifuged pellets were then washed three times with 1% Triton Buffer, and bound proteins were eluted by boiling in 1X SDS-PAGE loading buffer.

The antibodies used in the study were anti-Flag (Sigma, St. Louis, MO), HA (HA11, BAbCo, Richmond, CA), EGFR (1005, Santa Cruz), anti-EGFR (IMC-C225/ Erbitux, ImClone Systems, Branchburg, NJ), EGFR-pY992 (Millipore, Bellerica, MA), EGFR-pY1068 (R&D Systems, Minneapolis, MN), EGFR-pY1173 (Santa Cruz), ku70 (sc-5309, Santa Cruz), anti-Met (C-12, Santa Cruz), anti-proliferating cell nuclear antigen (PCNA, PC-10, Santa Cruz) and Rak (MAB3766, R&D Systems). For western blots of immunoprecipitation reactions of myc- and flag-tagged proteins, antibodies were biotinylated using the Amersham protein biotinylation system (GE Healthcare, Piscataway, NJ) and western blots were probed with horseradish peroxidase-conjugated streptavidin (Thermo Scientific) at a concentration of 125 ng/ml.

For kinase assays, immunoprecipitated kinases were washed twice with kinase buffer (10 mM HEPES, pH 7.4, 10 mM $MgCl_2$ and 1 mM DTT) and resuspended in 30 μl kinase buffer containing 30 μM ATP and 1 μg kinase substrate (GST-EGFR-1141-1211) and incubated at 30°C for 30 minutes. The reaction was terminated with SDS and boiling before analysis by western blot. To measure cell surface EGFR levels, cells were biotinylated with sulfo-NHS-SS-biotin and purified with avidin-agarose using the Cell Surface Isolation Kit (Thermo Scientific) according to the manufacturer's instructions. In some cases, proteins that remained unbound to the avidin-agarose served as a control for intracellular proteins.

Imaging- For EGFR immunofluorescence, cells were fixed with formaldehyde, permeabilized with Triton X-100 and stained as described previously (45). However, for Rak immunofluorescence, cells were permeabilized with methanol. EGFR was stained using the Ab-13 antibody (Thermo Scientific) and Rak was stained with a monoclonal antibody

(MAB3766, R&D). Images were captured on a Leica DM IRBE inverted microscope at the University of Kentucky Imaging Core Facility.

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FIGURE LEGENDS

Figure 1. Rak increases EGFR-Y1173 phosphorylation directly. (A) A549 NSCLC cells were infected with a control adenovirus (Ad-GFP, lane 1) or increasing doses of an adenovirus driving expression of Rak fused to an HA epitope tag sequence (lanes 2-4). Lysates were analyzed by western blot for HA-Rak (upper panel), total EGFR (second panel), EGFR-phosphoY1068 (third panel), EGFR-phosphoY1173 (fourth panel) and ku70 as a loading control (fifth panel). (B) NCI-H226 squamous cell lung cancer cells were infected with the same doses of the same viruses as in panel A and analyzed for the same proteins and phosphorylation sites. (C) HEK293 embryonic kidney cells were transfected with a control plasmid (lane 1) or a plasmid encoding Rak fused to a myc epitope tag (lane 2) and analyzed for the same protein and phosphorylation sites as described in panels A and B. (D) Ovcar-3 human ovarian cancer cells were transfected with a control siRNA (lanes 1 and 3) or an siRNA pool targeting Rak (lanes 2 and 4) and were untreated (lanes 1 and 2) or stimulated with 20 nM EGF for 5 minutes. Rak knockdown was confirmed by western blot (top), while EGFR was unaffected (second panel) and EGFR-pY1173 decreased (third panel, compare lanes 3 and 4). (E) A549 cells were transfected with a control plasmid (lane 1) or a plasmid encoding flag-tagged Rak (pcDNA3.1-Rak-flg, lane 2), and immunoprecipitated Rak

was analyzed by western blot (middle panel) or incubated in a kinase assay with a purified fusion protein of glutathione S-transferase (GST) fused to amino acids 1141-1211 of EGFR (lower panel, Coomassie staining). The kinase assay was then analyzed by western blot for EGFR-Y1173 (top panel). The lower band is a non-specific band caused by the antibody light chain in the precipitation reaction. (F) The same reactions as described in panel E were performed with immunoprecipitated EGFR (middle panel), resulting in negligible Y1173 phosphorylation (upper panel).

Figure 2. EGFR and Rak co-precipitate and co-localize. (A) HEK293 cells were co-transfected with a plasmid encoding EGFR and either a control plasmid (lane 1) or a plasmid encoding myc-tagged Rak (lane 2). Rak was immunoprecipitated using an anti-myc antibody, and precipitates were analyzed for EGFR (upper panel) or Rak (middle panel). The lower panel shows a western blot of cell lysates for EGFR. (B) HEK293 cells were co-transfected with plasmids encoding EGFR and Rak and precipitated with a control antibody (lane 1) or an anti-EGFR antibody (lane 2), and precipitates were probed for myc-Rak (top panel) or EGFR (lower panel). (C) A549 NSCLC cells were transfected with a control plasmid (lane 1) or a plasmid encoding myc-tagged Rak (lane 2). Rak was immuno-precipitated, and western blots were probed for EGFR (upper panel) and Rak (middle panel). The lower panel shows EGFR expression in cell lysates. (D) A549 cells were transfected with a plasmid encoding myc-tagged Rak and precipitated with a control antibody (lane 1) or an anti-EGFR antibody (lane 2). Precipitates were probed for myc-Rak (top panel) or EGFR (middle panel). (E) Rak (top) and EGFR (bottom) were detected by western blot in SW480 human colon cancer cells and A431 human carcinoid cells. (F) EGFR was immunoprecipitated (lane 2) from SW480 (top) or A431 cells (bottom) and analyzed by western blot for Rak or EGFR. Lanes 1 and 3 are immunoprecipitation reactions with control mouse immunoglobulin.

Figure 3. Extracellular EGFR levels decrease with Rak expression. (A) A549 cells were infected with a control adenovirus (A-B, lanes 1 and 3) or a matched virus encoding Rak (A-B, lanes 2 and 4). Cell surface proteins were biotinylated and purified by avidin-agarose. Extracellular proteins bound avidin (lanes 3-4) while intracellular proteins remained unbound (lanes 1-2). (A) Extracellular EGFR decreased following Rak expression (lanes 3 and 4), and the majority of Rak was detected in the intracellular pool (lanes 2 and 4). (B) PCNA was a marker of intracellular proteins and localized to the intracellular pool. The samples in panels C and D were the same as in panels A and B, lanes 3 and 4. (C) Extracellular Met levels were unchanged and the proportion of labeled proteins was not generally affected (D). (E) A bar graph represents the amount of extracellular, avidin-bound EGFR in cells from panel A, lanes 3 and 4. (F) Immunofluorescence for EGFR (using the Ab-13 antibody, see Methods), Rak (HA epitope tag) or a merged image (right) following infection with a control adenovirus (top panels) or a Rak adenovirus (lower panels). EGFR membrane localization (white arrowheads) decreased with Rak expression, and EGFR co-localized with Rak in intracellular punctate sites.

Figure 4. The Rak SH2 and SH3 domains cooperate to increase EGFR binding. (A) HEK293 cells were transfected with plasmids encoding EGFR and wild-type Rak (lanes 7 and 8) or Rak mutants Y497F (carboxy-terminal tyrosine, lanes 1 and 2), K262R (kinase-inactivating mutation, lanes 3 and 4) and deletion of both the SH2 and SH3 domains (lanes 5 and 6). Lysates were precipitated with control mouse immunoglobulin (odd lanes) or an antibody to EGFR (even lanes), and precipitated protein complexes were probed with antibodies to Rak (upper panel) or EGFR (second panel). The lower panels show western blots of Rak (third panel) and EGFR (bottom panel) expression in the lysates, where the lower migration reflects the deleted portions of the protein. The SH2 and SH3 domain

deletion caused a marked reduction in EGFR complex formation. (B) Quantitation of band intensities from panel A, reflecting bands from the upper panel divided by those in the second panel.

Figure 5. Rak-EGFR binding increases after receptor stimulation. (A) HEK293 cells were transfected with plasmids encoding EGFR and myc-tagged Rak and stimulated with 20 ng/ml EGF for increasing amounts of time. Lysates were then immunoprecipitated with a control antibody (lanes 1, 3 and 5) or an anti-EGFR antibody IMC-C225 (lanes 2, 4 and 6), and the precipitated proteins were analyzed by western blot for Rak (top) or EGFR (bottom). The blots showed that the EGFR-Rak complex increased in abundance following EGF stimulation and persisted for 30 minutes post-stimulation. There was a small decrease in Rak abundance relative to a ku70 loading control. (B) HEK293 cells expressing myc-tagged Rak and EGFR were untreated (lane 1) or treated with EGF (lane 2) for 5 minutes. Myc-Rak was analyzed by western blot (middle panel) or immuno-precipitated and tested in a kinase assay using EGFR-GST (lower panel) as a substrate, as in Figure 1. EGF treatment led to an increase in Rak activity in a western blot for EGFR-pY1173 (upper panel). The results from triplicate experiments are quantitated in the lower panel. (C) HEK293 cells were transfected with plasmids encoding EGFR, myc-tagged Rak and either a control vector (lane 1), flag-tagged Grb2 (lane 2), or a Grb2 inactivating R86K mutant (lane 3). Grb2 was immunoprecipitated (third panel), and EGFR (upper panel) and Rak (second panel) co-precipitated with wild-type Grb2, but not the R86K mutant. EGFR (fourth panel) and Rak (lower panel) expression was unchanged in the three reactions. (D) HEK293 cells were transfected with the same plasmids described in panel C, but lysates were precipitated with control antibodies (lanes 1 and 3) or antibodies to EGFR (lanes 2 and 4). Precipitated EGFR (third panel) associated with Rak (top panel) and wild-type Grb2 (second panel, lane 2). EGFR did not precipitate with mutant Grb2 (second panel, lane 4), and co-precipitating Rak

increased when mutant Grb2 was co-expressed (top panel, lane 4). Rak (fourth panel) and Grb2 (lower panel) expression was unchanged in the reactions. (E) In an affinity precipitation assay, lysates from HEK293 cells transfected with Rak were tested for binding to a column-bound GST fusion protein (lane 1) or GST-EGFR (lane 2). Rak binding to GST-EGFR was detected by western blot (upper panel), and the lower panel is a Coomassie-stained gel of the fusion proteins.

Figure 6. Elevated Rak binding to mutant EGFR. (A) MDA-MB-435 melanoma cells were transfected with a vector control (lane 1, vec) or plasmids encoding wild-type EGFR (lane 2, WT) or EGFR-Δ747 (lane 3, mut). Lysates were either western blotted for EGFR, phosphotyrosine, myc-tagged Rak or ku70 (as indicated) or precipitated with antibodies to EGFR and blotted for Rak or EGFR (as indicated). The ratio of precipitated Rak as a percent of input Rak is plotted below. (B) MDA-MB-435 cells were transfected with vector control (lane 1) or plasmids encoding EGFR-Δ747 (lane 2 and 5), EGFR-Δ747 plus Rak (lanes 3 and 6), or Rak alone (lanes 4 and 7) and were untreated (lanes 1-4) or stimulated with EGF for 5 minutes. Lysates were blotted for EGFR, ku70, Rak or EGFR-pY1068, as indicated. Results were quantitated and plotted below. (C) MDA-MB-435 cells were transfected with a plasmid encoding Rak and either a vector control (lanes 1 and 4) or plasmids encoding wild-type EGFR (lanes 2 and 5) or EGFR-Δ747 (lanes 3 and 6). Cells were biotinylated and purified as described in Figure 3. Intracellular proteins (lanes 1-3) included Rak and PCNA (lower two panels). EGFR-wild-type localized to the plasma membrane (lane 5), while mutant EGFR was not detectable at the extracellular surface when Rak was expressed (lane 6). (D) MDA-MB-435 cells were transfected with a vector control (lanes 1 and 4), a plasmid encoding EGFR-Δ747 (lanes 2 and 5) or plasmid encoding EGFR-Δ747 and Rak (lanes 3 and 6). Cells were biotinylated and purified as described in Figure 3. In the absence of Rak, EGFR-Δ747

localized to the plasma membrane (lane 5) and then disappeared from the extracellular surface when Rak was expressed (lane 6).

Supplemental figure 1. Rak expression decreased the phosphorylation of EGFR tyrosine 992. The samples are identical to those of Figure 1A and showed unchanged EGFR levels but decreased EGFR-Y992 phosphorylation.

Supplemental figure 2. Rak does not co-precipitate with Met but associates with endogenous EGFR. (A) HEK293 cells were co-transfected with a control plasmid (lane 1) or a plasmid encoding myc-tagged Rak (lane 2). Rak was immunoprecipitated using an anti-myc antibody, and precipitates were analyzed for Met (upper panel) or Rak (second panel). The lower panel shows a western blot of cell lysates for Met (third panel) and Rak (lower panel). (B) Rak (upper panel) and EGFR (lower panel) were endogenously expressed in HEK293 cells, although at lower levels than post-transfection. (C) HEK293 lysates were precipitated with a control antibody (lane 1) or an anti-EGFR antibody (lane 2), and precipitates were probed for endogenous Rak (top panel) or EGFR (lower panel).

Supplemental figure 3. Rak decreases plasma membrane EGFR levels in HEK293 cells. Rak and EGFR were transiently transfected into HEK293. Cell surface proteins were biotinylated and purified by avidin-agarose. Extracellular proteins bound avidin (lanes 3-4) while intracellular proteins remained unbound (lanes 1-2). Extracellular EGFR decreased following Rak expression (A, lanes 3 and 4), and the majority of Rak was detected in the intracellular pool (B, lanes 2 and 4). PCNA was a marker of intracellular proteins and localized to the intracellular pool (C). Quantitation of EGFR levels is shown in panel D.

Supplemental figure 4. Rak-EGFR binding does not require the presence of the Rak SH2 or SH3 domains independently. (A) HEK293 cells were transfected with plasmids encoding EGFR and wild-type Rak (lanes 5 and 6) or Rak mutants deleted in the SH2 and SH3 domains (lanes 1-2 and 3-4, respectively), and lysates were immunoprecipitated with a control antibody (lanes 1, 3 and 5) or an antibody to EGFR (lanes 2, 4 and 6). Precipitated protein complexes were probed with antibodies to Rak (upper panel) or EGFR (second panel). The lower panels show western blots of Rak (third panel) and EGFR (bottom panel) expression in the lysates, where the lower migration reflects the deleted portions of the protein. (B) Quantitation of band intensities from panel A, reflecting bands from the upper panel divided by those in the second panel. (C) A Rak kinase-inactive mutant does not increase EGFR-Y1173 phosphorylation. A549 cells were transfected with a control plasmid (lane 1, con) or plasmids expressing kinase-deficient Rak (lane 2, KD) or wild-type Rak (lane 3, WT) and analyzed for Rak (top panel), EGFR (second panel), EGFR-pY1068 (third panel), EGFR-pY1173 (fourth panel) or ku70 (lower panel).

Supplemental figure 5. Loading controls for Rak biotinylation experiments. (A) Each of the three biotinylated lysates from Figure 6C contained equivalent amounts of protein (upper panel), and EGFR expression was approximately equal by western blot (lower panel). (B) Analogous experiments as described in panel A for the samples in Figure 6D. (C) The panel shows a longer exposure of the western blot in panel 6C. The intensities of the EGFR bands in lanes 2 and 3 are approximately the same.

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