Therapeutic IMC-C225 Antibody Inhibits Breast Cancer Cell Invasiveness via Vav2-Dependent Activation of RhoA GTPase

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Abstract **Purpose:** Abnormalities in the expression and signaling pathways downstream of epidermal growth factor receptor (EGFR) contribute to progression, invasion, and maintenance of the malignant phenotype in human cancers. Accordingly, biological agents, such as the EGFR-blocking antibody IMC-C225 have promising anticancer potential and are currently in various stages of clinical development. Because use of IMC-C225 is limited, at present, only for treatment of cancer with high EGFR expression, the goal of the present study was to determine the effect of IMC-C225 on the invasiveness of breast cancer cells with high and low levels of EGFR expression.

Experimental Design: The effect of IMC-C225 on invasion was studied using breast cancer cell lines with high and low levels of EGFR expression.

Results: The addition of EGF led to progressive stress fiber dissolution. In contrast, cells treated with IMC-C225 showed reduced invasiveness and increased stress-fiber formation. Interestingly, IMC-C225 pretreatment was accompanied by EGFR phosphorylation, as detected using an anti – phosphorylated tyrosine antibody (PY99), which correlated with phosphorylation of Vav2 guanine nucleotide exchange factor and activation of RhoA GTPase irrespective of EGFR level, and Vav2 interacted with EGFR only in IMC-C225 – treated cells. The underlying mechanism involved an enhanced interaction between β1 integrins and EGFR upon IMC-C225 treatment.

Conclusion: Here, we defined a new mechanism for IMC-C225 that cross-links integrins with EGFR, leading to activation of RhoA and inhibition of breast cancer cell invasion irrespective of the level of EGFR in the cells, thus providing a rationale for using IMC-C225 in the metastatic setting independent of the levels of EGFR.

Epidermal growth factor receptor (EGFR) signaling regulates many cellular processes, including proliferation, differentiation, motility, and invasion, and imbalances in these cellular processes contribute to tumor formation and metastasis. Human breast cancer progression is often associated with aberrant up-regulation of EGFR, and members of the EGFR family are targets for several anticancer therapies, including breast cancer (1, 2). Structural analysis of EGFR in complex with the anti-EGFR antibody IMC-C225 revealed that interaction of IMC-C225 with the ligand-binding region of EGFR sterically prevents the receptor from dimerization and activation (3). This is believed to be a key mechanism of the antitumor effects of IMC-C225 antibody. However, the possibility remains that EGFR could be stabilized by IMC-C225, without ligand binding, through heterodimerization with other receptors, such as integrins, at the cell-cell contact sites (4).

Integrins are cell surface adhesive receptors composed of α and β subunits. Integrin-mediated adhesion stimulates multiple signaling pathways that modulate actin cytoskeleton organization, cell motility, and cell growth. The cytoplasmic domains of α and β subunits are devoid of enzymatic activity; however, through interactions with cytosolic proteins, such as focal adhesion kinase (FAK) and Src kinase, integrins are able to promote intracellular signaling. Integrins also cooperate with growth factor receptors, such as insulin-like growth factor-I receptor, hepatocyte growth factor receptor (c-Met), platelet-derived GFR (PDGFR), and EGFR, in assembling the transduction machinery (5). The EGFR-integrin association seems to be complex and continue to be poorly understood. Integrin α1β1 functions as a negative regulator of EGFR signaling through the activation of a protein tyrosine phosphatase TCPTP (6), whereas integrin αvβ3 acts as a positive regulator of EGFR activation (7). Integrins also regulate EGFR expression. Recent study by Reginato and colleagues showed that EGFR expression is regulated by β1 integrin and is reduced when the cells are detached from extracellular matrix (8). The cooperation between β1 integrin and EGFR signaling also regulate adhesion properties of a cell. Integrin can induce EGFR tyrosine phosphorylation in the absence of the ligand EGF, leading to activation of the EGFR pathway (9). Integrin functions are also complemented by EGFR. In keratinocytes, activated EGFR phosphorylates α6β4 and disrupts hemidesmosomes

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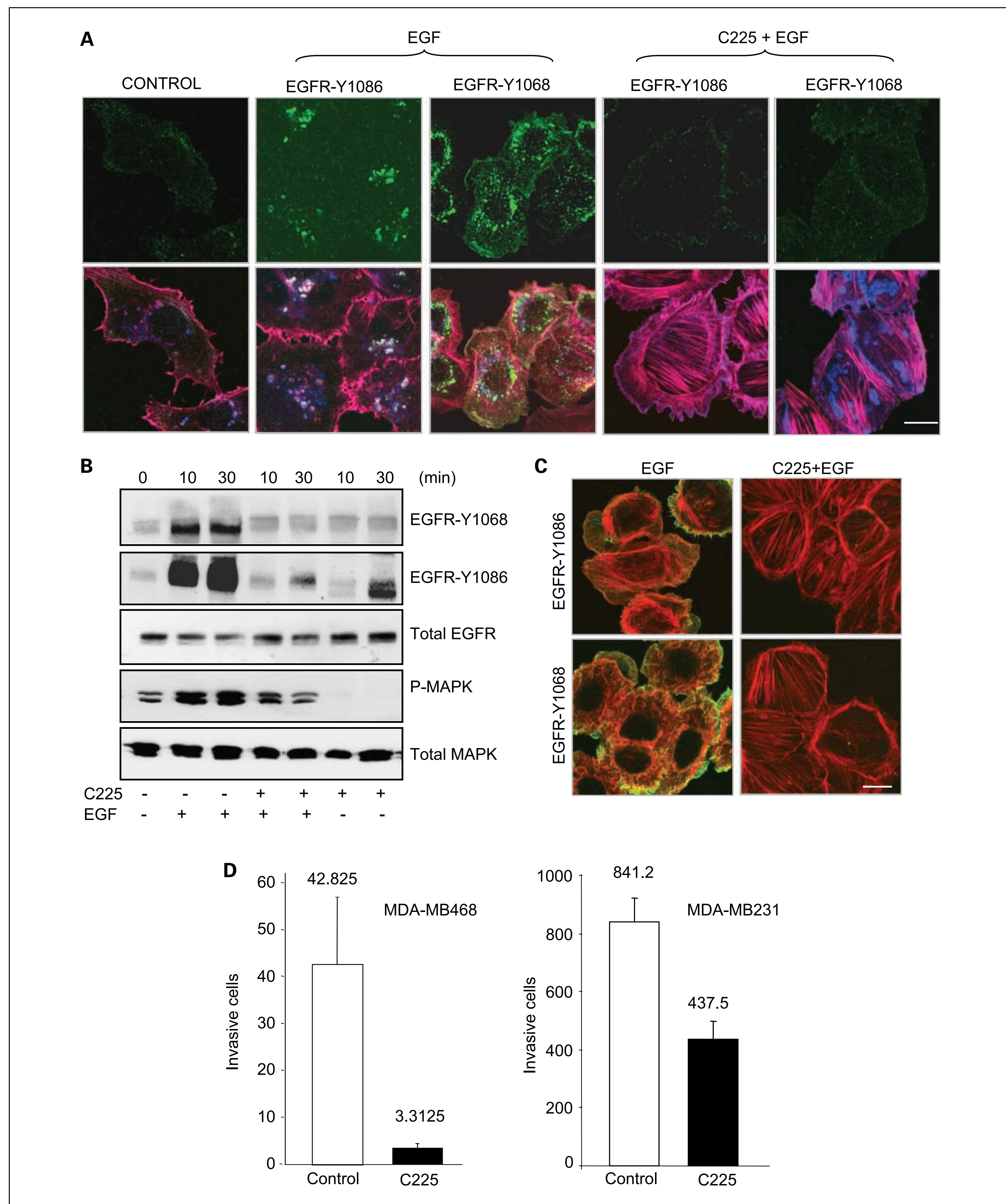

Fig. 1. Cytoskeleton rearrangement and modulation of the signal transduction pathway by IMC-C225. *A,* representative immunofluorescence image showing distribution of phosphorylated EGFR (Y^{1086} and Y^{1068}, *green*) and total pools of EGFR (*blue*) in MDA-MB468 cells in the presence or absence of EGF and IMC-C225. F-actin is shown in red. *B,* MDA-MB468 cells treated with EGF for various time points in the presence or absence of IMC-C225 and processed for immunoblotting using specific antibodies. Western blot showing specific inhibition of EGFR and mitogen-activated protein kinase (*MAPK*) phosphorylation by the blocking antibody IMC-C225. *C,* distribution of phosphorylated EGFR (Y^{1086} and Y^{1068}, *green*) in MDA-MB231 cells in the presence or absence of EGF and IMC-C225. F-actin is shown in red. Bar, 20 μm. *D,* bar plot showing inhibition of migration of MDA-MB468 and MDA-MB231 cells on treatment with IMC-C225.

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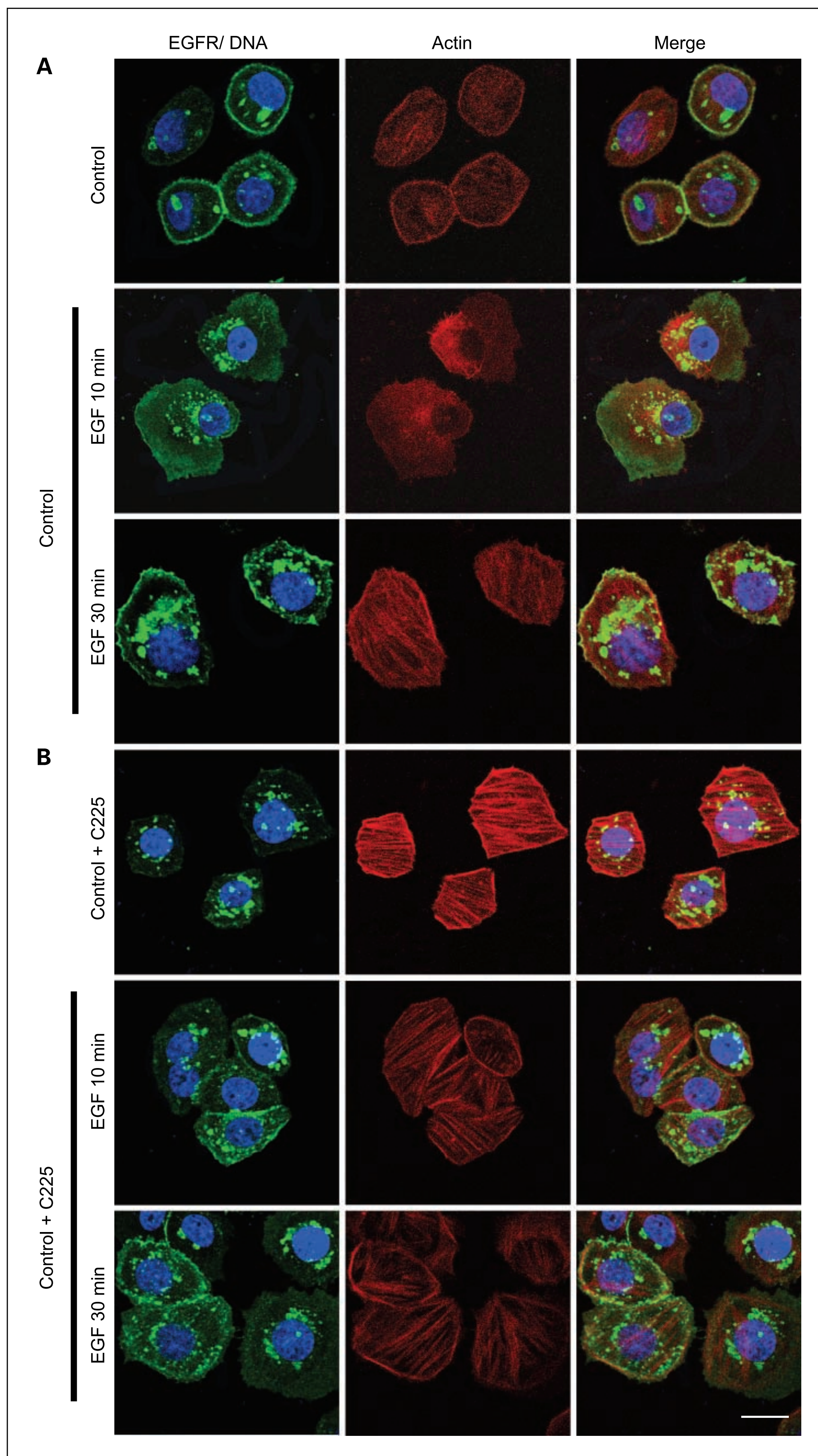

Fig. 2. Intracellular trafficking of EGFR. Representative immunofluorescence images showing distribution of total EGFR in response to EGF stimulation for 10 and 30 min in control (*A*) and in IMC-C225– pretreated (*B*) MDA-MB468 cells. EGFR (*green*), F-actin (*red*), and DNA (*blue*). Bar, 20 μm.

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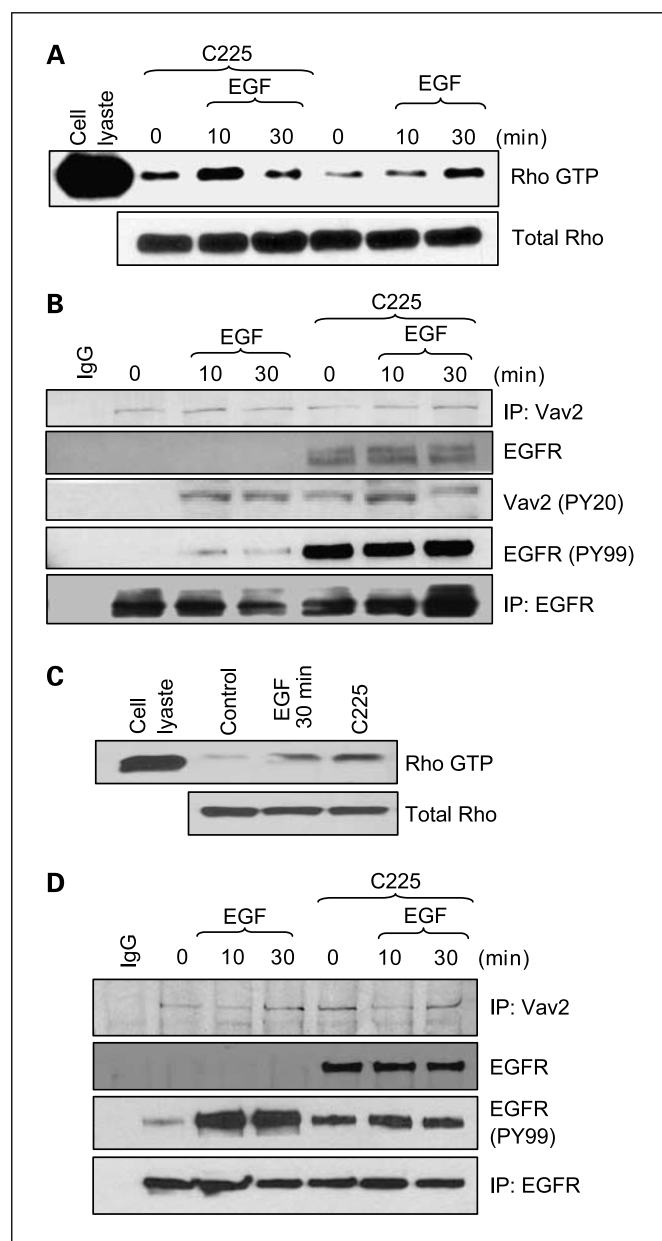

Fig. 3. Effect of IMC-C225 on RhoA activation. MDA-MB468 cells (*A*) or MDA-MB231 cells (*C*) were serum-starved or pretreated with IMC-C225 before stimulation with EGF. Western blot showing levels of Rho GTP and total RhoA. *B,* Vav2 immunoprecipitated from MDA-MB468 cells treated as mentioned above (*first panel*) and probed for EGFR (*second panel*) and phosphorylated Vav2 (PY20, *third panel*). Total EGFR immunoprecipitated (*last panel*) and probed for phosphorylated EGFR using anti – phosphorylated tyrosine antibody (PY99, *fourth panel*). IgG was used as negative control. *D,* Vav2 immunoprecipitated from MDA-MB231 cells treated as mentioned above and probed for EGFR (*first panel*). Immunoblot showing interaction of Vav2 with EGFR in IMC-C225 – treated cells independent of EGF stimulation (*second panel*). EGFR was immunoprecipitated from MDA-MB231 cells (*last panel*) treated as mentioned above and probed for phosphorylated EGFR (PY99). Immunoblot showing increased levels of phosphorylated EGFR (PY99) in MDA-MB231 cells treated with either EGF or IMC-C225 compared with untreated cells (*third panel*).

by activating Fyn, which leads to increased metastasis (10, 11). In all, activation of EGFR correlates with changes in cell morphology and increased migration and invasiveness of cell lines and increased invasiveness of tumors *in vivo* (12 – 14).

Metastasis involves invasion of tumor cells through tissue and extracellular matrix to distant site, and this process requires a complex interplay of signaling from receptors to intracellular molecules, such as mitogen-activated protein kinase, lipid kinases, phospholipases, Ser/Thr and Tyr kinases, scaffold proteins, and Rho GTPases. The Rho family of GTPases is ubiquitously expressed and acts as molecular switch that control signal transduction pathways by cycling between a GDP-bound inactive form and a GTP-bound active form. In the GTP-bound state, GTPases interact with downstream targets to elicit a variety of intracellular responses, such as regulation of gene expression and NADPH oxidase activity, actin cytoskeleton dynamics, and cell cycle progression (15). The best-characterized function of the Rho family members Cdc42, Rac, and RhoA is the regulation of actin-based structures known as filopodia, lamellipodia, and stress fibers, respectively (16). In a migrating cell, the spatiotemporal activation of Rho GTPase is not well understood, but accumulating evidences show a role for integrins, as well as EGFR, in activation of Rho GTPases, Rac1 and RhoA (17, 18). Both β1 and β3 integrins play a role in the formation of lamellipodia and stress fibers and promote dramatically different modes of migration on fibronectin via differential activation of Rho GTPases (19). In human corneal epithelial cells, LPA-stimulated EGFR induces activation of Rac and Rho GTPases, whereas in breast cancer cells, overexpression of RhoA induces EGFR activation and increases migration (20). In leukocytes, Rho activation promotes migration (21). On the contrary, in fibroblasts, activation of Rho leads to inhibition of cell migration (22). Although EGFR activation is considered as a potent stimulus for cell migration, the relationship and the hierarchy between EGFR and Rho GTPases is poorly understood and seems to be cell type – specific and stimulus-specific.

Because use of IMC-C225 is limited, at present, only for treatment of cancer with high EGFR expression, the goal of the present study was to determine the effect of IMC-C225 – induced EGFR blockade on cell motility using a breast cancer cell model with low and high EGFR levels. The addition of EGFR-blocking antibody to the cells increased stress fibers in the cells independent of the levels of EGFR and induced phosphorylation of Vav2, which in turn stimulated its downstream RhoA GTPase, leading to the formation of stress fibers. Activation of Vav2 was determined to be a consequence of increased EGFR phosphorylation, resulting from the formation of a complex between β1-integrins and EGFR in response to IMC-C225 treatment. Here, we showed that IMC-C225 can promote interactions between integrins and EGFR, leading to EGFR activation in the absence of ligand and stimulate RhoA GTPase to inhibit the migration of breast cancer cells.

Materials and Methods

Antibodies. Antibodies to β-catenin, Src, COOH terminus of EGFR, glyceraldehyde-3-phosphate dehydrogenase, PY99, RhoA (Santa Cruz Biotechnology, Inc.), NH_2 terminus of EGFR, CD44, PY20 (Neomarkers), phosphorylated EGFR antibodies (Biosource), phosphorylated mitogen-activated protein kinase, phosphorylated Src, total mitogen-activated protein kinase (Cell Signaling), Vav2 (Abcam), and β1 integrin (Chemicon Intl.) were used in the present study. Antimouse and antirabbit antibody conjugated to Alexa 488 and Alexa 546, Topro-3, and TRITC-labeled phalloidin were purchased from Molecular Probes, Inc.

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Cell culture and treatment. MDA-MB468 and MDA-MB231 breast cancer cells were obtained from the American Type Culture Collection. The cells were cultured in DMEM/F12 with 10% serum. When mentioned, cells were serum starved for 24 h and then followed by treatment with EGF at 100 ng/mL with or without pretreatment with IMC-C225. IMC-C225 was kindly provided by ImClone Systems, Inc. Src inhibitor PP2 (Calbiochem) was used at 40 μmol/L for 30 min when indicated.

Indirect immunofluorescence. For colocalization studies, cells were cultured on coverslips, fixed in methanol or 4% paraformaldehyde, and then blocked in 10% nonimmune goat serum in PBS for 30 min at room temperature. Primary antibodies at 1:500 dilution were applied to coverslips for 2 h at room temperature. After washes with PBS, fluorescently tagged secondary antibodies were applied for 1 h at the room temperature. Topro-3 and TRITC-labeled phalloidin was used to counterstain DNA and polymerized actin, respectively. Confocal microscopic analyses were done using an Olympus FV300 laser scanning confocal microscope (Olympus, Olympus America, Inc.) in accordance with established methods.

Subcellular fractionation. MDA-MB468 cells were serum starved for 48 h, stimulated with EGF (100 ng/mL) in the presence or absence of IMC-C225, then transferred to 4°C, and washed with cold PBS. The plasma membrane fraction was separated from the cytosol fraction using sucrose gradient as described earlier (23).

Reporter gene assays. Cells were maintained in DMEM supplemented with 10% FCS. Transient transfections were done using the Fugene-6 system (Roche Molecular Biochemicals). In brief, exponentially growing cells were cultured in six-well plates and transfected with 0.8 μg of TopFlash reporter construct (Millipore Corp.) and 0.02 μg of pCMV β-galactosidase control. At 6 h posttransfection, the cells were transferred to medium containing 1% FCS. After 36 h, the cells were treated with EGF at various time intervals, as indicated, with or without IMC-C225 pretreatment (1 h). Transfection efficiency was normalized through β-galactosidase activity. Each assay was done at least thrice in triplicate sets.

Transfection. MDA-MB468 cells were transfected with either V14 RhoA (dominant active, DA) or V12 Rac (DA) using the Fugene-6 system (Roche Molecular Biochemicals) according to manufacturer's protocol.

Migration assays. To measure the effect of antibody on cell migration, MDA-MB468 or MDA-MB231 cells were either serum starved for 48 h or serum starved and pretreated with IMC-C225. Cells were trypsinized and resuspended in serum-free medium in the presence of 0.1% bovine serum albumin and loaded on the upper well of Boyden chamber at a concentration of 100,000 per well in the presence or absence of IMC-C225. The lower side of the chamber was filled with conditioned medium of NIH-3T3 fibroblasts with 0.1% bovine serum albumin. After 12 h, the filters were removed, fixed, and stained using H&E. The results are represented as a percentage of the total cells that had migrated through the filter.

Rho and Rac1 activation assays. The Rho activation and Rac1 activation assays were done using glutathione *S*-transferase–tagged Rhotekin-RBD and Pak-PBD protein beads, respectively (Cytoskeleton, Inc.), according to the manufacturer's protocol.

Gel electrophoresis, immunoprecipitation, and Western blotting. All experiments were done in triplicate, and the data shown were representative of all three sets of experiments. Cellular extracts were processed, as previously described (24).

Results and Discussion

Breast cancer cells MDA-MB468, treated with EGF (100 ng/mL) for 30 min with or without IMC-C225 (50 nmol/L)

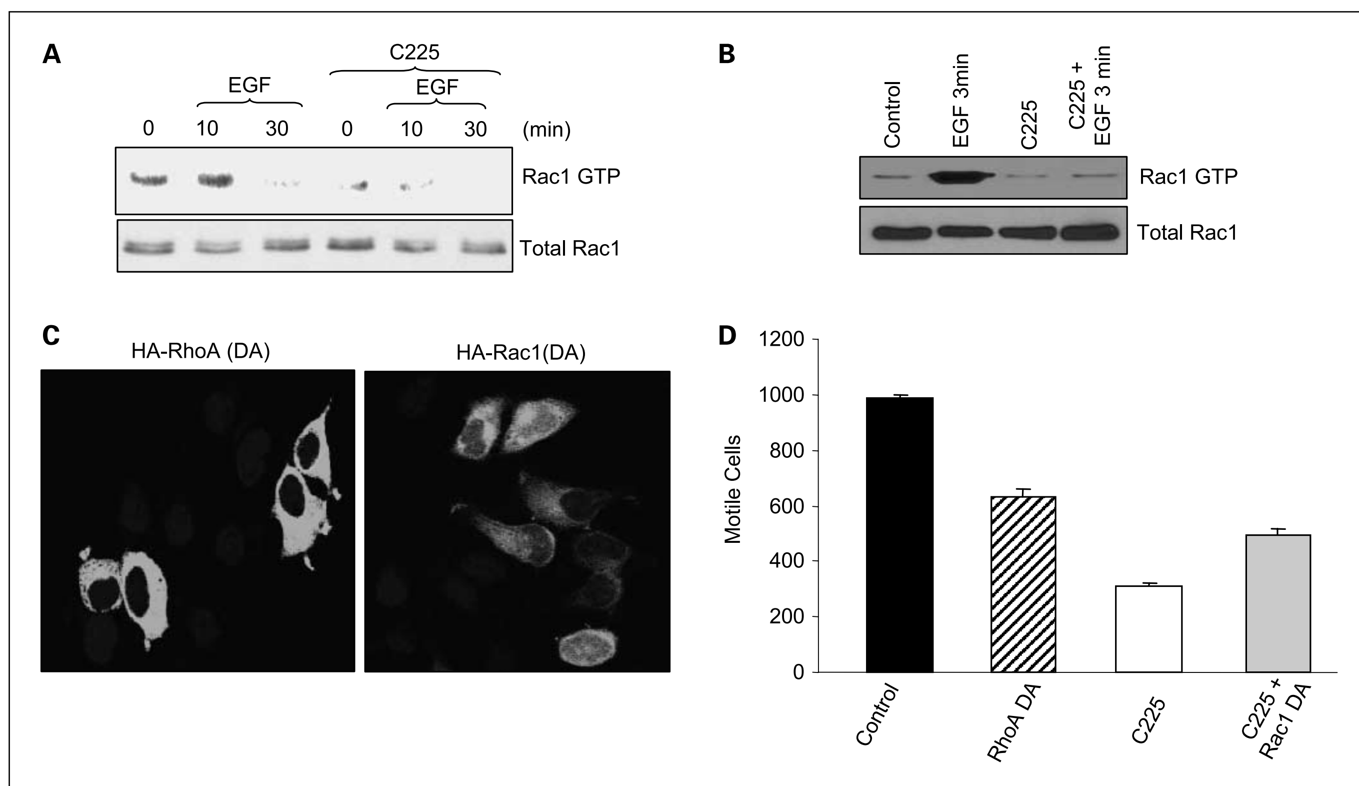

Fig. 4. *IMC-C225–mediated activation of Rac1.* MDA-MB468 cells were serum-starved and pretreated with IMC-C225 before stimulation with EGF. Western blot showing levels of Rac1 GTP and total Rac1 for longer time points (*A*) and shorter time points (*B*) of EGF stimulation. *C,* immunofluorescence image showing transfection of myc-tagged V14 RhoA (DA, *green*) or V12 Rac (DA, *green*) and DNA (*blue*) in MDA-MB468 cells. *D,* bar plot showing effect of V14 RhoA and V12 Rac transfection on migration of MDA-MB468 in presence or absence of IMC-C225 pretreatment.

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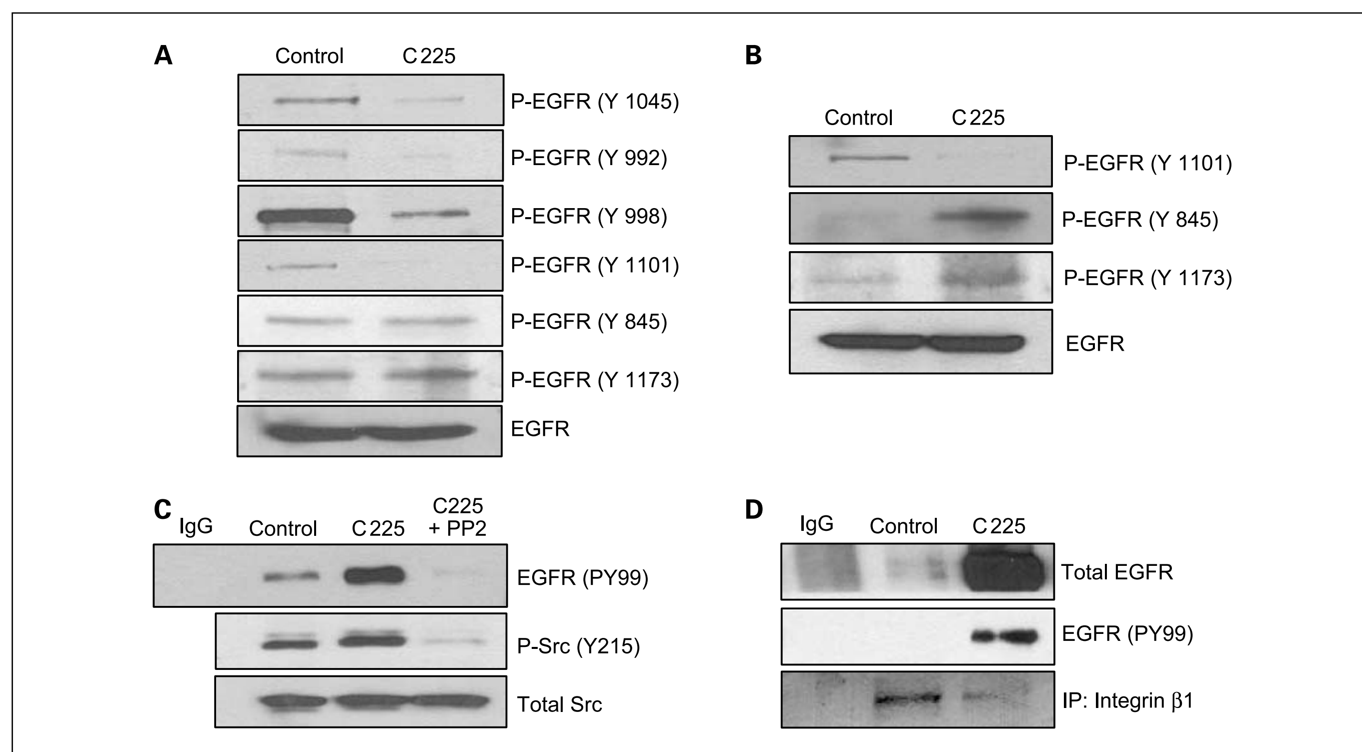

Fig. 5. IMC-C225 – mediated activation of EGFR via integrins. Immunoblot showing effect of IMC-C225 treatment on EGFR phosphorylation at various tyrosine residues in MDA-MB468 (*A*) and MDA-MB231 cells (*B*). Total EGFR was used as an internal control. *C,* EGFR was immunoprecipitated from MDA-MB231 cells after treatment with IMC-C225 or IMC-C225 and Src inhibitor PP2 and probed with phosphorylated EGFR antibody. Western blot showing effect of Src inhibitor PP2 on Src phosphorylation on Y^{215}. Total Src was used as an internal control. *D,* immunoblot showing EGFR in a complex with β1 integrin only in response to IMC-C225 treatment in MDA-MB468 cells. EGFR immunoprecipitated with β1 integrin in response to IMC-C225 treatment was phosphorylated on PY99.

pretreatment, were stained for filamentous actin (F-actin), total EGFR, and active EGFR (Y^{1086} and Y^{1068}) using appropriate antibodies. An increased amount of inactive EGFR was localized on the membrane or in the cytoplasm in unstimulated cells, and EGF stimulation resulted in EGFR phosphorylation at both the Y^{1086} and Y^{1068} sites. F-actin was prominent around the periphery in the membrane ruffles of the cells under basal, as well as EGF-stimulated conditions (Fig. 1A). On the other hand, antibody blockage led to aggregation of EGFR under the cellular surface, suggesting antibody-mediated internalization of EGFR, which was confirmed using subcellular fractionation studies. Serum-starved MDA-MB468 cells showed majority of the EGFR on the plasma membrane, but both EGF-treated and IMC-C225 – treated cells showed a small easily detectable fraction of EGFR in the cytosol fraction along with membrane localization of EGFR. The purity of fractionation was confirmed using CD44 and glyceraldehyde-3-phosphate dehydrogenase as markers for plasma membrane and cytosol, respectively (Supplementary Fig. S1). IMC-C225 treatment also led to complete abolishment of phosphorylation of EGFR at Y^{1086} and Y^{1068} (Fig. 1A and B) as expected. IMC-C225 – treated cells showed increased formation of stress fibers with no change in the pattern of actin distribution on EGF stimulation (Fig. 1A). Next we studied the effect of IMC-C225 on EGFR phosphorylation in E-cadherin – negative and low EGFR – expressing MDA-MB231 cells. IMC-C225 also inhibited EGFR phosphorylation at both Y^{1086} and Y^{1068} sites and promoted the formation of stress fiber in MDA-MB231 cells (Fig. 1C). Because EGFR-initiated cytoskeletal modifications are known to regulate invasive phenotype, we next studied the effect of IMC-C225 antibody on invasion. IMC-C225 treatment significantly inhibited invasion of both MDA-MB468 ($P < 0.005$) and MDA-MB231 ($P < 0.04$) cells, but the inhibition was to a greater extent in MDA-MB468 cells, which express higher levels of EGFR compared with MDA-MB231 cells (Fig. 1D). In brief, IMC-C225 inhibited the invasion of cells independent of its EGFR status.

EGF treatment of MDA-MB468 cells dissociates actin, α-actinin, and vinculin from the E-cadherin – catenin complex, and this activity coincides with a robust phosphorylation of β-catenin, plakoglobin/γ-catenin, and p120 (cas) on tyrosine residues (25, 26). EGF-mediated signal transduction also involves localization of several SH2 domain – bearing proteins, including c-Src, to the plasma membrane that culminates in the regulation of cellular processes, such as cell motility (27 – 29). To gain insight about the signal transduction pathways affected by EGFR blocking antibody, EGF-treated MDA-MB468 cells with or without antibody pretreatment were stained for c-Src phosphorylation at tyrosine 215 (Y^{215}). IMC-C225 treatment completely blocked Src phosphorylation on Y^{215} in MDA-MB468 cells (Supplementary Fig. S2). In addition, the antibody treatment efficiently blocked the mitogen-activated protein kinase activation, as detected using Western blot analysis (Fig. 1B). In brief, IMC-C225 inhibited activation of classic signaling complexes in MDA-MB468 cells but resulted in a profound stimulation of stress fibers, suggesting the potential involvement of an alternate pathway.

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After activation, EGFR is known to get internalized through the endocytic pathway where EGF binding is abolished at the endosomal pH of ~5 (30). Release of the receptor from its ligand can, in turn, activate a series of signaling pathways. However, it has been suggested that IMC-C225 binding to soluble EGFR remains unchanged when pH is reduced from 7 to 5, which precludes ruling out the possibility of activation of signaling from the endocytic compartment (3). MDA-MB468 cells were treated with EGF with or without pretreatment with IMC-C225, and its effect on actin dynamics was assessed over time (Fig. 2A and B). Interestingly, serum-starved cells (control) showed stress fibers only in response to EGF treatment; however, IMC-C225 pretreatment was sufficient to induce stress fibers, and the F-actin distribution remained unchanged in response to EGF (Supplementary Figs. S3 and S4). Therefore, IMC-C225 was able to modulate cytoskeleton organization in breast cancer cells.

Numerous studies have shown that β-catenin plays a dual role in both cell-cell adhesion and cell proliferation, depending on its location. Membrane-associated β-catenin stabilizes cadherin-mediated adhesion by facilitating the cytoskeletal attachment of adhesion complexes. In contrast, nuclear-associated β-catenin activates several genes important in cell proliferation and invasion (31–33). Interestingly, EGFR forms a complex with β-catenin (34). Therefore, we next investigated the effect of IMC-C225 treatment on β-catenin posttranslational modifications. MDA-MB468 cells were treated with EGF with or without IMC-C225 pretreatment and costained for F-actin and β-catenin. After 25 min of EGF treatment, there was a significant nuclear translocation of β-catenin (Supplementary Fig. S5A, *top panel and its corresponding inset*). In contrast, IMC-C225 pretreatment was capable of blocking this translocation, as β-catenin was detected in a cytoplasmic pool or at the tight junctions (Supplementary Fig. S5A, *bottom and its corresponding inset*). The finding that IMC-C225 blocked β-catenin–inducible gene activation was confirmed by using the β-catenin–specific TopFlash reporter construct (Supplementary Fig. S5B). Cell motility is a complex process involving cell-substrate interactions, as well as cell-cell interaction, and the process involves EGFR phosphorylation of a number of cytoskeleton proteins, including FAK. Phosphorylation of FAK through EGF signaling is an important step in modulating cell-substrate interactions during cell migration (35). To determine whether FAK is one of the targets in the antimigratory effect of the blocking antibody, we monitored the changes in FAK distribution (Supplementary Fig. S5C). Upon EGF treatment, there was a gradual dissolution of the abundantly present focal adhesion complexes that initially excluded the cell periphery (5 min) and progressively disengaged after the first 15 min. A total dissolution of these mature focal complexes with formation of small focal points situated at the base of lamellipodia and leading edges was detected 30 minutes after the addition of EGF, when the cells displayed an evident tendency to scatter (Supplementary Fig. S5C). In contrast, pretreatment with the EGFR-blocking

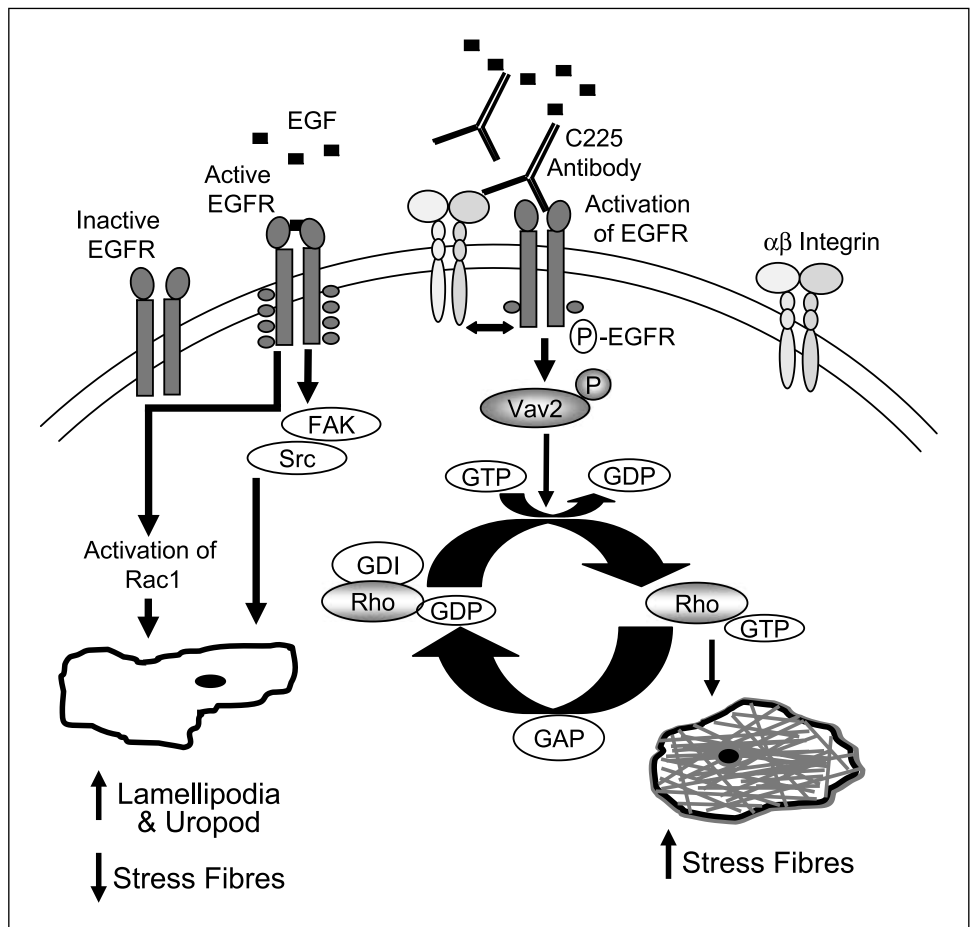

Fig. 6. Proposed model of activation of Rho GTPase caused by IMC-C225–mediated cross-linking between β1 integrins and EGFR on the cell membrane leading to inhibition of cell migration.

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antibody showed increased FAK, indicating increased adhesion or reduced motility.

In response to stimulus, two distinct types of cytoskeleton reorganization have been observed—one involving actin stress fibers and another involving accumulation of actin filaments forming membrane ruffles (36). In addition, focal adhesions are formed at points of stress fibers terminating at the plasma membrane. Actin stress fiber formation and focal adhesion assembly are induced by Rho activation, whereas membrane ruffling requires Rac proteins (37). Because IMC-C225 treatment induces stress fiber formation and increased focal adhesion, we next studied the status of RhoA activation in response to IMC-C225. In serum-starved MDA-MB468 and MDA-MB231 cells, Rho GTP levels increased in response to EGF stimulation. In contrast, cells pretreated with IMC-C225 exhibited increased levels of active RhoA independent of EGF stimulation (Fig. 3A and C, respectively).

Three classes of regulatory proteins affect the activation state of Rho GTPases. Guanine nucleotide exchange factors promote the exchange of GDP for GTP to activate the GTPase. GTPase-activating proteins negatively regulate by enhancing its intrinsic GTPase activity, and guanine nucleotide dissociation inhibitors block the GTPase cycle by sequestering and solubilizing the GDP-bound form (16). The Vav family of guanine nucleotide exchange factors activates Rho GTPases (Rho, Rac, and Cdc42) by catalyzing the exchange of GDP for GTP. The three mammalian Vav proteins (Vav1, Vav2, and Vav3) differ in their tissue distribution. Vav1 is predominantly expressed in hematopoietic cells, whereas Vav2 and Vav3 are ubiquitously expressed (38). To understand the mode of RhoA activation in our model system, we immunoprecipitated Vav2 and studied its activation status in both control and IMC-C225–pretreated MDA-MB468 cells with or without EGF treatment. Interestingly, phosphorylated Vav2 (using PY20 antibody) was detected in IMC-C225–pretreated cells independent of EGF stimulation. In contrast, in control cells, phosphorylated Vav2 was detected only after EGF stimulation. These finding suggest that IMC-C225 treatment was sufficient to activate Vav2 (Fig. 3B). To gain further insight into the activation of Vav2, we probed the same blot with the anti-EGFR antibody. We found Vav2 formed a complex with EGFR only in the presence of IMC-C225. Because IMC-C225 inhibited phosphorylation of EGFR on Y^{1086} and Y^{1068}, we next studied EGFR phosphorylation using an anti–phospho tyrosine antibody (PY99). Control cells showed phosphorylation of EGFR only in response to EGF stimulation. However, intense phosphorylation of EGFR was observed in IMC-C225–pretreated cells, and EGF stimulation did not affect the phosphorylation levels any further (Fig. 3B). EGFR activation and interaction with Vav2 in response to IMC-C225 treatment was also observed in MDA-MB231 cells (Fig. 3D). In brief, IMC-C225–activated EGFR, through tyrosine residues other than Y^{1068} and Y^{1086} and this process, was independent of ligand stimulation. In addition, in IMC-C225–treated cells, phosphorylation of Vav2 could be a consequence of direct interaction with activated EGFR, but in EGF-treated control cells, because Vav2 failed to form a complex with phosphorylated EGFR, its activation seems to be an indirect event.

Although both EGF and IMC-C225 treatment led to RhoA activation, only EGF treatment increased cell invasion whereas IMC-C225 treatment inhibited cell invasion. Recent studies show role for Rac1 stimulation in response to increased phosphorylation of EGFR with subsequent activation of Vav2 (17, 39–41). To understand role for Rac1 in IMC-C225–mediated inhibition of cell invasion, pull-down assays using the PBD of PAK were done to measure Rac1 activation in MDA-MB468 cells treated with either EGF or with IMC-C225 for the same time points as studied for RhoA activation. Only EGF-treated cells exhibited slight increase in Rac1 GTP levels at 10 min followed by decrease at 30 min of stimulation (Fig. 4A). Because Rac1 activation is an early event, we focused on earlier time point of stimulation. Dramatic increase in Rac1 activation was observed for only EGF-treated cells (3 min of EGF treatment), whereas IMC-C225 treatment did not show significant change in Rac1 stimulation during this time (Fig. 4B). This indicated that EGF stimulation leads to an early activation of Rac1, which trigger cell invasion, but IMC-C225 treatment leads to RhoA activation and thereby inhibition of cell invasion. To confirm this, we transfected MDA-MB468 cells with DA RhoA or DA Rac1 and studied its effect on cell invasion in the presence or absence of IMC-C225. RhoA (DA)–transfected cells showed decreased invasion (~ 40%) compared with control cells, whereas IMC-C225–treated cells transfected with Rac1 (DA) showed increased invasion (~ 40%) compared with untransfected IMC-C225–treated cells (Fig. 4D). The transfection efficiency was studied by staining for the myc tag on the transfected Rho GTPases (Fig. 4C). Although IMC-C225 treatment leads to Vav2 phosphorylation, what precludes Rac1 from being activated and triggers activation of only RhoA is not known at this point and requires further studies.

Several studies have shown that either inhibition or constitutive activation of RhoA decreases cell motility (42, 43). In human corneal epithelial cells, activation of RhoA promotes wound healing (44). Similarly, overexpression of the wild-type human RhoA in tumoral mesothelial (MM1) cells increases the invasive capacity both *in vitro* and *in vivo* (45). Recent study by Mateus et al. showed that E-cadherin–dependent EGFR activation leads to RhoA activation and enhanced cell motility (46). In contrast, in the present study, we show IMC-C225–dependent EGFR activation leads to RhoA activation and inhibition of breast cancer cell motility. This is similar to the observed inhibition of migration in fibroblasts on activation of Rho and ROCK (22). In brief, our noted differences with those of Mateus et al. (used hamster Chinese hamster ovary cells) could be use of two different cell types. In conclusion, the EGFR-RhoA pathway is complex and seems to be cell type–specific and stimulus-specific, and future studies are needed to unravel the complex regulation of cell migration by growth factor receptors.

Systematic analysis of stimulant-dependent EGFR phosphorylation using mass spectrometry has revealed multiple potential phosphorylation sites; however, antibodies are available only to a few of these sites. MDA-MB468 cells were screened for phosphorylation on various potential tyrosine residues (Y^{1045}, Y^{992}, Y^{998}, Y^{1101}, Y^{845}, and Y^{1173}) in response to IMC-C225 treatment (Fig. 5A). Unfortunately, in MDA-MB468 cells, IMC-C225 treatment did not enhance phosphorylation at any of the tyrosine residues studied. In contrast, in MDA-MB231 cells, IMC-C225 treatment enhanced phosphorylation on Y^{845} (Fig. 5B). Because activation of EGFR on Y^{845} is known to involve c-Src kinase, we next studied the effect of the c-Src inhibitor PP2 on EGFR phosphorylation in response to

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IMC-C225 treatment. IMC-C225 – pretreated MDA-MB231 cells treated with PP2 showed loss of Src, as well as EGFR phosphorylation, as detected using antibody to Y^{215} and PY99 antibody, respectively (Fig. 5C), thus confirming a role for c-Src in IMC-C225 – mediated activation of EGFR on Y^{845} in MDA-MB231 cells.

To understand the mechanism of EGFR activation by IMC-C225, we focused on alternate signaling pathways that activate EGFR independent of EGF stimulation. Integrin α1β1-EGFR cross-talk plays a key role in negatively regulating Rac1 activation (39). Moreover, recent studies have revealed dynamic association of integrins α2β1 with EGFR in the absence of growth factors (9). Similarly, in the present study, we found that β1 integrins formed a complex with EGFR, but only in IMC-C225 – treated cells (Fig. 5D). The association of integrins with growth factor receptors is suggested by coclustering and direct coprecipitation studies (47 – 49). Earlier studies have shown that when A431 cells are treated with cross-linking agents, EGFR is coprecipitated with integrins α2 and β1, indicating a weak interaction between integrins and EGFR (4). Interestingly, as opposed to the previous studies, we found that IMC-C225 forms a stable complex between integrins and EGFR that could be precipitated using Triton-X solution. Therefore, our results suggest a novel mechanism wherein IMC-C225 – mediated inhibition of cell invasion in MDA-MB 468 and MDA-MB231 cells (Fig. 6), involve cross-linking between integrins and EGFR, leading to activation of Vav2 guanine nucleotide exchange factor and subsequent activation of RhoA leading to the formation of stress fibers irrespective of the EGFR levels in breast cancer cells.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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